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QUANTITATIVE PROBLEMS IN BIOCHEMISTRY

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BY

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FOREWORD

In the course of the last twenty years or so Biochemistry has evolved from the descriptive and qualitative stage into a more mature phase in which it ranks as probably the most exact of all the sciences included in the field of Biology. This process of evolution has necessitated a change in the outlook and training of the student who wishes to make Biochemistry his speciality. Whereas in the past it was sufficient for him to take as his main ancillary subjects physiology and organic chemistry, he is now compelled to have more than a nodding acquaintance with physical chemistry as well and to be able to understand how the principles of physical chemistry can be applied to biological systems.

This situation in itself creates a pedagogic problem, for the number of teachers of physical chemistry who have the necessary biological background to appreciate the biochemist's problems is still all too small. Consequently any attempt made to help the student of biochemistry to a better understanding of physico-chemical problems deserves the most vigorous encouragement.

Students of chemistry have available to them a number of books dealing with chemical calculations, but no equivalent volume dealing with biochemical problems has until now been on the market, although several texts on physical biochemistry have been published during the last few years. This volume by Dr. Dawes should therefore be of considerable value in filling a gap in modern biochemical education.

Dr. Dawes has had wide experience in teaching senior students of biochemistry working for the Honours Degree in Glasgow, and at an earlier date in Leeds, and his compilation of a sufficient range of numerical problems has been a task which has spread over several years. Each group of problems is gathered into a chapter containing enough explanatory text to give the fundamental information required for their solution without at the same time converting the volume into a full fledged textbook of physical biochemistry.

Although the book has been designed to meet the specific needs of senior students of biochemistry, it may also have an

appeal to workers in allied fields who are anxious to keep their knowledge of biochemistry up to date. However this may be, it is certain of a very warm welcome from members of the Honours Biochemistry Class in the University of Glasgow, and, it is hoped, by students taking the equivalent courses in other universities.

J. N. DAVIDSON.

THE UNIVERSITY
GLASGOW.
1956.

PREFACE

'When you can measure what you are speaking about and express it in numbers, you know something about it, and when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind.'—*Lord Kelvin.*

In the past the biochemist has been at a disadvantage compared with his colleagues in the more chemical and physical sciences because much of his work was, by its very nature, of a qualitative rather than a quantitative kind. Consequently the biochemist was regarded with something akin to suspicion by his colleagues in these fields, a suspicion perpetuated by the remark of that great physical chemist G. N. Lewis, when he accused living things of being 'cheats in the game of entropy'; clearly the investigator of such phenomena was himself something of an enigma!

During the past two decades the advances made in physical biochemistry and biophysics have completely revolutionized the quantitative approach to living matter and we are now in the position of having a wide field of data upon which to draw for numerical problems. Furthermore, living things are now known to 'play the game' with entropy.

This book is the outcome of a policy pursued by the author, initially at Leeds and later at Glasgow, of including numerical problems in the examinations for the Honours Degree in Biochemistry. The author feels that the greatest benefit to be derived from numerical problems is the attitude of mind engendered. All too frequently one encounters a tendency for students of biochemistry, after receiving their initial training in chemistry and physics, to become rather illogical and imprecise in thought if their course, apart from certain sections of the practical work, provides little quantitative expression. Numerical problems, properly applied, can help to combat this danger. They can also afford a clearer understanding of experimental techniques that are not usually encountered in the undergraduate laboratory course. For instance, not every biochemical laboratory possesses an analytical ultracentrifuge, and a better appreciation of the measurements that must be made before a molecular weight can be calculated is obtained if actual sample calculations are carried out.

The original plan was to assemble a collection of suitable problems alone, but it was then felt that the book might prove to be of more value to the student if some theoretical background and worked examples were included, especially since the literature on the various topics treated is somewhat diffuse and there is no single textbook which provides the required coverage. As the intention was for emphasis to be placed on problems rather than on the development of a textbook of physical biochemistry, the greatest difficulty besetting the author was the attempt to achieve a suitable balance between inclusion and exclusion of material; the pitfalls of such a course are patently obvious. Choice of material has been quite deliberate in order to keep the book within reasonable compass, and some basic knowledge is assumed. However, to compensate for any deficiency resulting from this treatment, references and suggestions for further reading are included at the end of each chapter.

It is not intended that the chapters should necessarily be worked through in a given order; each chapter is reasonably self-contained and cross-references are provided where necessary. Many of the problems have either been taken or constructed from data in published papers and, in these instances, the references are cited. This enables the student to consult the original publication should difficulty arise. Some attempt has been made to provide problems of roughly graded difficulty in order that the book might be of use not only to honours students but also to those taking biochemistry to principal or subsidiary level, corresponding to the Double and Single Science courses of the University of Glasgow.

It is a pleasure to record my gratitude to several friends who have assisted during the preparation of this book. Professor J. N. Davidson has offered constant encouragement and advice throughout, and kindly consented to write a Foreword. I am greatly indebted to my former colleagues Dr. S. Dagley and Mr. T. J. Bowen, both of whom have given generously of their time to read the entire manuscript, and who not only criticized freely but also contributed many valuable suggestions which have been incorporated in the text. Helpful suggestions in connexion with certain portions of the text were also made by Dr. H. N. Munro, Dr. W. C. Hutchison, Mr. W. H. Holms and Dr. J. C. Speakman.

Through the kindness of Professor Eric G. Ball, Dr Fred Richards and Dr Frank Gurd of Harvard University Medical School I have been able to include some problems devised for use in the Harvard Medical Sciences 201 ab course. Many of the diagrams are the work of Mr. R. Callander, for whose help I am extremely grateful. Thanks are due to authors and publishers for permission to reproduce certain diagrams, and to Mr. T. J. Bowen for Figures 7.1 and 7.2. Answers to some of the problems were kindly checked by Mr. A. Fleck and Dr. Shelagh M. Foster, but I alone must accept responsibility for errors which may still remain. I should also like to thank the authors and publishers of the papers quoted in this book for permission to use their data. Mr. W. H. Holms, Mr. D. Murray and my wife all rendered valuable assistance with proof reading, and their help is greatly appreciated. Mr. C. Macmillan and Mr. J. Parker, of Messrs E. & S. Livingstone Limited, have given kindly guidance and have helped in many ways to facilitate the production of the book. Finally, but by no means least, I am indebted to my wife, who, starting as a novice, finished as a skilful typist in the preparation of the manuscript for the printer.

E. A. D.

Glasgow, 1956.

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APPENDICES

LOGARITHMIC NOTATION

The following convention with regard to logarithms is employed throughout this book.

$$\log = \log_{10}$$
$$\ln = \log_e$$

They are related by the expression :

$$\ln x = 2{\cdot}303 \log x$$

TEMPERATURES

Unless stated to the contrary, all temperatures recorded are in degrees Centigrade.

CHAPTER I

DETERMINATION OF MOLECULAR WEIGHTS

The molecular weight of substances of small molecular size may be determined by measurement of the colligative properties of their solutions. These include the elevation of boiling point, depression of freezing point and of vapour pressure of the pure solvent, and osmotic pressure. Many compounds of biological origin, such as proteins and polysaccharides, are macromolecules and have very large molecular weights. They can be studied only in very dilute solution; this point is made clear by an example. Molecular weight determinations of small molecules are usually carried out in 0·01-0·1 M solution. A solution 0·01 M with respect to a protein having a molecular weight of 75,000 would have to contain 750 grams of the protein per litre, and this is a physical impossibility. The most highly soluble proteins (certain albumins) will not dissolve to this extent in water, and even if this were possible to achieve, the resulting solution would be quite unsuitable for molecular weight determination. This is because the colligative properties of solutions depend on the number of molecular units present per unit volume and not on their size. Consequently a very small amount of a compound of low molecular weight may exert an effect equal to or even greater than that of the protein itself. Of the colligative properties, only osmotic pressure is used for such determinations, because in this case, by suitable choice of conditions, the effect of small molecules or ions associated with the protein may be eliminated. It is clear, therefore, that in general, to determine the molecular weights of macromolecules, other methods must be employed. These may be divided into two main groups:

1. Those based on the chemical composition, by analysis of certain elements or amino acids, or by combining weights.

2. Physico-chemical methods which include sedimentation, diffusion, viscosity, flow birefringence and light scattering. Osmotic pressure also comes under this heading. These methods find the widest application at the present time.

1. Calculation of Molecular Weight from Chemical Composition.

(*a*) Elementary and Amino Acid Analysis

The molecular weight of a compound such as a protein may be calculated if its elementary composition is known. Since a compound must contain in its molecule at least one atom of every element shown by analysis to be present, the mass of the compound which contains one gram-equivalent of such an element will be the minimal molecular weight. In other words, the molecular weight cannot be smaller than this amount. The compound may contain more than one atom of the element, and the molecular weight is then an integral multiple of the minimal value based on the assumption that only one atom is present. Thus, if the percentage of the element present in the compound is known, the minimal molecular weight is given by the expression:

$$\text{Minimal molecular weight} = \frac{\text{atomic weight of element}}{\text{percentage of element in compound}} \times 100 \quad . \quad . \quad (1.1)$$

and true molecular weight

$$= n \times \text{minimal molecular weight} \quad . \quad . \quad (1.2)$$

where n is the number of atoms of the element present in the molecule.

Example 1.1.—The nitrogen content of serine is 13·33 per cent. Calculate the minimal molecular weight.

$$\text{Min. mol. wt.} = \frac{14}{13{\cdot}33} \times 100 = 105.$$

Serine contains one nitrogen atom, so that $n = 1$ and the true molecular weight is also 105.

Example 1.2.—Lysine contains 19·17 per cent. nitrogen. What is the minimal molecular weight?

$$\text{Min. mol. wt.} = \frac{14}{19{\cdot}17} \times 100 = 73.$$

Lysine contains two nitrogen atoms and therefore the true molecular weight is 146.

To obtain the true molecular weight it is necessary, therefore, to know the value of n. This is usually obtained by some other

method such as the measurement of one of the colligative properties of a solution of the compound. As the value of n increases, its evaluation becomes more difficult. This is because the value for the minimal molecular weight becomes correspondingly smaller, until, eventually, it may be within the range of experimental error in the measurement of the colligative property being used to determine n. For accuracy it is necessary to select an element which is present in the molecule in a relatively small amount and which can be determined with accuracy.

Further information may be obtained if analytical figures are available for more than one element which is present in the compound. For instance, if in Example 1.1 the additional information had been given that serine contains 45·71 per cent. oxygen, we should have been able to obtain the minimal molecular weight of $\frac{16}{45{\cdot}71} \times 100 = 35$ from the oxygen data and 105 from the nitrogen value. If the serine molecule contains n_1 atoms of nitrogen and n_2 atoms of oxygen, then the molecular weight of serine must be represented by the equation

$$n_1 \times 105 = n_2 \times 35.$$

This is satisfied by $n_1 = 1$ and $n_2 = 3$, and hence the molecular weight is 105 or some multiple of it. The data given do not permit a decision on the latter point, and in the absence of further evidence the molecular weight might be 210 or 315.

For the determination of the molecular weight of proteins, the elements normally used are sulphur and, in the case of metalloproteins, the metal present in the prosthetic group. The minimal molecular weight of haemoglobin, for example, has been determined by analysis of its iron content. The sulphur present in a protein may exist as disulphide, sulphydryl or thio-ether, and the sulphur determination may be expressed in these terms or simply as the percentage total sulphur. Elements such as carbon and nitrogen are present in too large a percentage for minimal molecular weight determinations and they give very large values of n.

Some proteins do not contain any element in sufficiently small quantity to be used in these calculations, but in many cases the percentage content of certain amino acids has been used for the determination of the minimal molecular weight. The procedure

is exactly analogous to that used when the basis is the elementary composition. Choice is made of an amino acid present in small percentage; the minimal molecular weight must contain at least one of these amino acid molecules. Provided the amino acid analysis is accurate, this method offers advantages, because, unlike an element, it is not likely that an amino acid will be present as a contaminant of the protein. Amino acids finding greatest application in this way are tyrosine, tryptophan and cystine, for they are usually present in small amount and can be accurately determined.

There are many data now available on the composition of various proteins, but unfortunately not all of them are reliable for the calculation of minimal molecular weights because of uncertainty in the state of purity of the protein analysed. There is also the possibility that in hydrolysing the protein prior to analysis some of the amino acids may be destroyed.

(*b*) Combining Weights

Minimal molecular weights may be determined as the weight of the compound which combines or reacts with 1 gram-molecule of a suitable chemical reagent such as a monovalent acid or base. Proteins, for example, contain a number of free carboxyl and amino groups in their molecules and these may be titrated with base and acid respectively. In this way the maximal base and acid-binding capacities are determined. If there are, say, x carboxyl groups per molecule, then one gram-molecule of protein will combine with x equivalents of base and the minimal molecular weight will be $1/x$ of the true molecular weight. x may be evaluated in a similar manner to n as described in the preceding section. The main disadvantage of this method is the large number of free acidic and basic groups which are present in most protein molecules and which thus yield very small minimal molecular weights with attendant difficulties in the evaluation of x.

Titration with acidic and basic dyes has also been used to determine the combining weights of proteins, and in the special case of respiratory proteins, combination with oxygen has been accurately measured (see Problem 1.6).

Example 1.3.—The maximal acid-combining capacity of egg albumin is $8{\cdot}7 \times 10^{-4}$ equivalents per gram protein. Calculate the minimal molecular

weight. The molecular weight of this protein, as determined by diffusion and sedimentation velocity measurements, is 43,800. Determine the approximate number of basic groups per molecule.

$8{\cdot}7 \times 10^{-4}$ equivalents of acid combine with 1 g. protein

$\therefore$ 1 equivalent of acid combines with $\dfrac{1}{8{\cdot}7 \times 10^{-4}}$

$= 1149$ g. protein.

Hence the minimal molecular weight is 1149.

The number of basic groups per egg albumin molecule will therefore be

$$\frac{43800}{1149} \simeq 38.$$

(*c*) End-group Analysis

This method presupposes a known or postulated structure of the molecular units on which basis the results are interpreted. Not unnaturally, this constitutes a major limitation to its usefulness. Furthermore, where a linear chain type of structure is assumed, any branching, unless quantitatively assessed, will introduce error. The classical example of end-group analysis is the estimation of the chain length of cellulose by Haworth and his collaborators. Assuming that cellulose consists of linear chains of glucose molecules joined by 1 : 4 linkages, the cellulose is completely methylated by the gentlest possible means and then hydrolysed under conditions which permit breakage of glucose-glucose links but not hydrolysis of the methyl groups. At one end of each cellulose chain will be a 2,3,4,6 tetramethyl glucose molecule, whereas all the other members of the chain will be 2,3,6 trimethyl glucose units. Accordingly analysis of the products of hydrolysis permits an estimate of the chain length. In this way the value of 100 to 200 β-glucose units, corresponding to a molecular weight of 20,000 to 40,000, was obtained. A further difficulty encountered in this work is the possibility of degradation of the chain during the methylation procedure so that the values must be regarded as minimal limits.

2. Physico-chemical Methods.

These are based on molecular kinetic theory and fall into two main categories depending on (1) the colligative properties of solutions, i.e. dependent on the *number* of molecular units present per given volume and (2) the *weight* of the units present. The number-average methods include measurement of osmotic pressure, spread monolayers, as well as end-group assay, whilst

weight-average methods embrace sedimentation, diffusion, viscosity and light-scattering.

The number-average molecular weight, M_n, is given by the expression

$$M_n = \frac{\Sigma n_1 M_1}{\Sigma n_1} = \frac{\Sigma C_1}{\Sigma n_1} \quad . \quad . \quad . \quad (1.3)$$

and the weight-average value, M_w, by

$$M_w = \frac{\Sigma n_1 M_1^2}{\Sigma n_1 M_1} = \frac{\Sigma C_1 M_1}{\Sigma C_1} \quad . \quad . \quad . \quad (1.4)$$

where n_1 is the number of molecules of molecular weight M_1, and C_1 is their concentration, equal to $n_1 M_1$.

The number-average and weight-average molecular weights afford information as to the dispersity of high molecular weight substances in solution. If a protein consists of molecular units all of the same size, it is said to be *monodisperse* and the molecular weight determined by number-average and weight-average methods will be the same. But if it consists of molecular units of different sizes, i.e. if it is *polydisperse*, the determined molecular weights are not the same, the weight-average figure always being greater than the number-average value. The ratio of these averages is a rough indication of the *polydispersity* of the protein.

Example 1.4.—The composition of a protein corresponds to 5 moles of molecular weight 15,000 and 10 moles of molecular weight 30,000.

The number-average molecular weight

$$= \frac{5 \times 15000 + 10 \times 30000}{5 + 10} = 25{,}000,$$

and the weight-average molecular weight

$$\frac{5 \times (15000)^2 + 10 \times (30000)^2}{5 \times 15000 + 10 \times 30000} = 27{,}000.$$

Molecular weight determinations on proteins are complicated by the shape and the electrical charge of the molecules. The former factor has a profound effect on molecular movement such as that measured in sedimentation and diffusion studies. Spherical molecules behave in a normal manner under these experimental conditions, but elongated, thread-like molecules of fibrous proteins deviate from the normal due to increased

frictional and also hydration effects; as a consequence their rate of diffusion is reduced.

The possibility of aggregation of molecules is increased in concentrated solution. Colligative phenomena, with the exception of osmotic pressure, are of little value for the determination of the molecular weights of macromolecules. Not only are the elevation of boiling point and depression of freezing point or vapour pressure too small to be measured, but, as already mentioned, the presence of traces of low molecular weight compounds, such as salts, would produce an effect equal to or even greater than that of the macromolecule itself. By correct choice of conditions the effect of such compounds can be eliminated in osmotic pressure measurements, the basis of which is now described.

(*a*) Osmotic Pressure

If a solution is separated from the pure solvent by a membrane permeable to molecules of solvent but impermeable to those of solute, then solvent molecules pass through the membrane into the solution until equilibrium is reached. The pressure which must be exerted on the solution to prevent the passage of solvent molecules across the membrane is a measure of the osmotic pressure of the solution. Osmotic pressure was first studied by the botanist Pfeffer in 1877, which perhaps emphasizes the importance of the phenomenon in biological systems. Van't Hoff showed that for very dilute solutions

$$\Pi V = RT \quad . \quad . \quad . \quad (1.5)$$

where Π is the osmotic pressure, V the volume containing 1 gram-molecule of solute, R the gas-constant and T the absolute temperature. Alternatively,

$$\Pi = CRT \quad . \quad . \quad . \quad . \quad (1.6)$$

where C is the concentration of solute in moles per litre. The value of R is 0·082 when Π is expressed in atmospheres and V in litres. The molar concentration C is equal to c/M, where c is the concentration in grams per litre and M the molecular weight of the solute. Thus:

$$M = \frac{cRT}{\Pi} \quad . \quad . \quad . \quad (1.7)$$

and by measuring the osmotic pressure of a solution of known concentration at a given temperature the molecular weight of the solute may be obtained. The van't Hoff equation holds only for very dilute solutions, however, and in practice it is often found that the osmotic pressures determined experimentally are considerably below those computed from equation 1.7. Now, since $\Pi/c = RT/M$, it follows that Π/c (sometimes called the

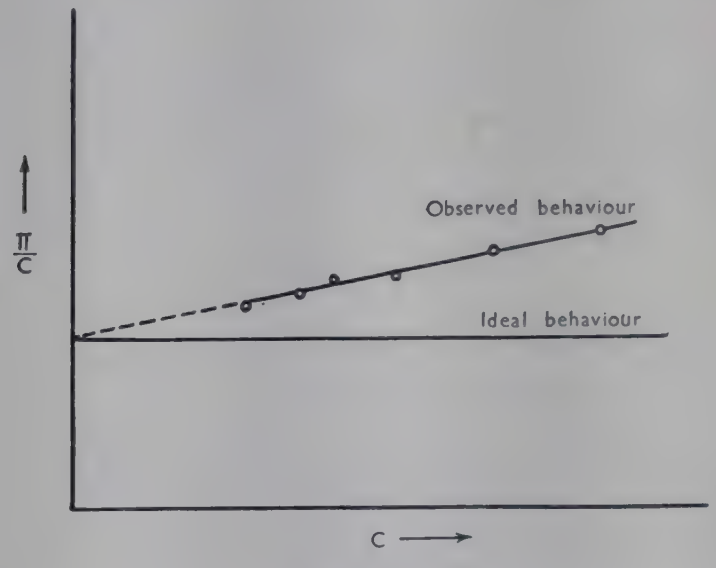

FIG. 1.1

Determination of the osmotic pressure Π at infinite dilution, by extrapolation to zero concentration, for molecular weight calculation.

reduced osmotic pressure) should be constant for all concentrations if the solution behaves ideally. Accordingly, Π/c is measured at several different concentrations and then Π/c is plotted as a function of c. In many cases this gives an almost linear curve which may be extrapolated to zero concentration, i.e. infinite dilution, and the Π/c intercept thus obtained used in the van't Hoff equation to evaluate M. This is shown in Fig. 1.1. The slope of the line is a measure of the interaction between solute and solvent and becomes greater when the solvent has a large solvating effect. It is also dependent on the shape of the molecule; the greater the asymmetry, the greater is the deviation from ideal behaviour because the elongated molecules, by solvation, immobilize solvent molecules.

With protein solutions there is the additional complication of the Donnan equilibrium (p. 43) whereby the free diffusion of inorganic ions through the membrane is restricted. Here the

observed osmotic pressure is produced both by protein molecules and inorganic ions. This arises because proteins are ampholytes and their ionization is affected by pH; on the acid side of their isoelectric point they exist as cations and on the alkaline side as anions. The Donnan effect may be minimized or even eliminated by making osmotic pressure measurements at the isoelectric point of the protein, but difficulty is often encountered because the protein displays its minimal solubility at its isoelectric point. Thus the observed osmotic pressure of protein solutions is dependent on the pH and is higher in acid and alkaline solution than at the isoelectric point. Also, to reduce the Donnan effect and to eliminate errors caused by traces of salts present in the protein solution, a concentrated salt solution is often used as solvent for the protein and a salt solution of the same concentration is placed on the other side of the semi-permeable membrane. Suitable corrections can be made for the ion effect in these cases.

A difficulty encountered in the determination of molecular weights of macromolecules by osmotic pressure measurements is the long periods of time required for the attainment of equilibrium. There is a danger that proteins may be either denatured or contaminated by bacterial growth. To overcome this time factor a dynamic osmometer has been devised in which the rate of flow of the solvent through the membrane, in response to a varied external pressure on the solution side of the membrane, is measured. By plotting the rate of flow against the external pressure and extrapolating to zero rate of flow, the osmotic pressure is obtained. It is equal to the intercept on the pressure axis. An example of this type of experiment is given in Problem 1.25.

To summarize, the determination of molecular weights from osmotic pressure measurements involves the following assumptions:

1. The system is at equilibrium.
2. The membrane is permeable to solvent molecules but not to solute molecules.
3. The osmotic pressure is proportional to the concentration of the solute (van't Hoff's Law).
4. The molecules of the solute are all of the same size.

With aqueous solutions of proteins and using collodion membranes, assumptions 1 to 3 are normally justified, subject to the provisions already discussed, but 4 may or may not be true and cannot be ascertained by osmotic measurements. It can be decided only by sedimentation and diffusion measurements. Finally, highly purified proteins must be used if the results are to be of any value.

Example 1.5.—A solution containing 0·388 g. of a sugar per 100 ml. water exerted an osmotic pressure of 380 mm. Hg at 10° C. Determine the molecular weight of the sugar.

$$M = \frac{cRT}{\Pi}$$

$$\Pi = 380 \text{ mm.} = \frac{380}{760} \text{ atm.}$$

$$T = 283°$$

$$c = 3{\cdot}88 \text{ g. per litre}$$

$$M = \frac{3{\cdot}88 \times 0{\cdot}082 \times 283 \times 760}{380} = \underline{180.}$$

Example 1.6.—The osmotic pressure of horse haemoglobin in 0·2 M phosphate was measured by Gutfreund at various concentrations of protein. The following results were obtained at 3° C.:

Haemoglobin concentration (g./100 ml.)	0·65	0·81	1·11	1·24	1·65	1·78	2·17	2·54
Osmotic pressure (cm. H_2O)	2·5	3·1	3·9	4·7	5·7	6·8	8·3	8·9
Haemoglobin concentration (g./100 ml.)	2·98	3·52	3·90	4·89	6·06	8·01	8·89	
Osmotic pressure (cm. H_2O)	11·2	13·4	14·6	19·6	23·9	34·2	38·7	

Use these data to determine the molecular weight of haemoglobin.

(After GUTFREUND (1949), p. 197, in *Haemoglobin*, ed. Roughton & Kendrew. London: Butterworth.)

This problem is solved graphically by plotting the reduced osmotic pressure Π/c against the concentration c. The values of Π/c are worked out as follows and then plotted in Fig. 1.2:

c	0·65	0·81	1·11	1·24	1·65	1·78	2·17	2·54
Π/c	3·84	3·82	3·51	3·79	3·46	3·82	3·82	3·40
c	2·98	3·52	3·90	4·89	6·06	8·01	8·89	
Π/c	3·76	3·80	3·74	4·00	3·94	4·27	4·36	

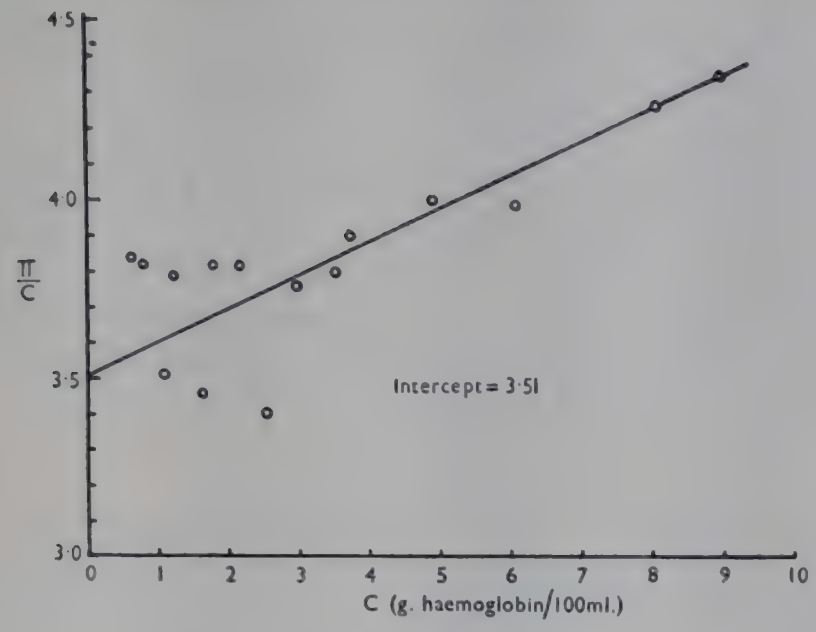

FIG. 1.2

The best straight line drawn through the experimental points is extrapolated to zero concentration and the intercept on the Π/c axis, which is equal to 3·51, is used in equation 1.7 to evaluate M. Note particularly that since Π is expressed in cm. H_2O the appropriate value of the gas constant R must be used. This is 8·471 × 10 litre cm. H_2O per mole per degree (see Appendix 2), and since c in equation 1.7 refers to grams per litre, whereas the concentrations given are in grams per 100 ml., the value obtained for Π/c must be divided by 10 to obtain the correct units, i.e. Π/c becomes 0·351.

$$\text{Hence } M = \frac{8{\cdot}471 \times 10 \times 276}{0{\cdot}351} = \underline{66{,}600.}$$

(*b*) SEDIMENTATION

Svedberg, in 1925, invented the ultracentrifuge, an instrument which enables very high centrifugal fields of up to 500,000*g* to be attained. If solutions of macromolecules are subjected to such fields, the molecules sediment owing to their large mass. Their concentration therefore increases from the centre of the centrifuge to the periphery. The tendency to sediment is opposed by the diffusion of the protein back from the highly concentrated region in the periphery to the more dilute region in the centre. At relatively low speeds an equilibrium is reached, when sedimentation is exactly opposed by the diffusion, and the distribution of protein in the centrifuge tube is then in a steady state. This occurs after a fairly long period of time and is the basis of the *sedimentation equilibrium* method of determining molecular weights. Alternatively, if high centrifugal fields are employed, the rate of sedimentation of macromolecules is greatly

in excess of their rate of diffusion and the latter factor becomes of minor importance. This is the *sedimentation velocity* method. These two techniques will now be treated separately.

Sedimentation Equilibrium.—At equilibrium the increment ds of solute driven in time dt by the centrifugal force in the direction of the periphery through unit surface is exactly the same as the increment moved by diffusion in the opposite direction. By equating and integrating the expressions for sedimentation and diffusion increments the following expression for the molecular weight of the solute may be obtained

$$M = \frac{2RT \ln (c_2/c_1)}{\omega^2(1 - \bar{v}\rho)(x_2^2 - x_1^2)} \quad . \quad . \quad (1.8)$$

where M is the molecular weight of the solute, R the gas constant in mechanical units, T the absolute temperature, c_2 and c_1 the concentrations of solute at distances x_2 and x_1 from the centre of rotation, ω the angular velocity[1], $\bar{v}$ the partial specific volume (this is the increment in volume when 1 gram of dry solute is added to a large volume of solution and for most proteins has a value near 0·74) and ρ the density of the solution.

Thus, at equilibrium and if the solute is monodispersed, measurement of the relative concentrations of solute at two distances from the centre of rotation enables the molecular weight to be evaluated. Inhomogeneity with respect to molecular weight will be revealed by differences in the value of M if a series of values of c are determined as a function of x.

The great disadvantage of this method is the long period of time required to attain equilibrium.

Sedimentation Velocity.—This is the more widely used method. It consists of the measurement of the rate at which macromolecules move in centrifugal fields of such force that the process of sedimentation greatly exceeds that of free diffusion. The solute molecules, which move outward from the axis of rotation, form a fairly sharp boundary between the solution and the pure solvent, and observation of the rate of movement of this boundary forms the basis of the method. The movement of the boundary is followed by changes in the refractive index and is observed by means of a Schlieren cylindrical lens system similar to that used in electrophoretic measurements. Movements are

[1] Expressed in radians per second. See Appendix 4.

recorded photographically at given intervals of time. When the solute molecules are moving with constant velocity, the centrifugal force is being balanced by the frictional resistance of the medium.

The rate of sedimentation is usually expressed in terms of the *sedimentation constant* s, which is the velocity for unit centrifugal field of force and has dimensions of time. From observation of the rate of boundary movement, s can be calculated from the formula

$$s = \frac{dx}{dt} \cdot \frac{1}{\omega^2 x},$$

where x is the distance from the centre of rotation and ω the angular velocity in radians per second. For proteins studied to date, s has values lying between 1 and 200×10^{-13} sec. A sedimentation constant of 10^{-13} sec. is termed one Svedberg unit (S) so that a value of 6×10^{-13} would be denoted by $6S$.

A solute in a centrifugal field sediments with a centrifugal force per gram-mole of $M(1 - \bar{v}\rho)\omega^2 x$ exactly opposed by the frictional force per gram-mole $F dx/dt$. The molar frictional coefficient F is usually obtained from the diffusion coefficient D by the relationship $F = RT/D$. Hence the molecular weight from sedimentation velocity and diffusion measurements is obtained by equating the centrifugal and frictional forces, and is given by the expression

$$M = \frac{RT}{D(1 - \bar{v}\rho)} \cdot \frac{dx}{dt} \cdot \frac{1}{\omega^2 x}$$

$$M = \frac{RTs}{D(1 - \bar{v}\rho)} \quad . \quad . \quad . \quad (1.9)$$

It will be noticed that an independent value for the diffusion coefficient is required. D is defined as the quantity of material diffusing per second across a surface area of 1 cm.^{2} when the concentration gradient is unity. Its measurement is discussed in the next section. ρ in equation 1.9 is usually taken as the density of the solvent and not of the solution although there is no universal agreement about this. In actual practice the distinction is not important.

Example 1.7.—Diphtheria toxin has been subjected to ultracentrifugal analysis and the following data obtained at 20°. The average sedimentation constant was $4{\cdot}6 \times 10^{-13}$ sec. and the average diffusion coefficient $5{\cdot}96 \times 10^{-7}$ cm.^{2}/sec. These analyses were carried out on solutions of the protein at various concen-

trations over the range 0·38 to 0·61 per cent. The density of water at 20° is 0·998. Obtain a value for the molecular weight of diphtheria toxin. The toxin has a partial specific volume of 0·736.

(After PETERMANN & PAPPENHEIMER, Jr. (1941), *J. phys. Chem.*, **45**, 1.)

The data given are substituted in equation 1.9.

$$M = \frac{RTs}{D(1 - \bar{v}\rho)}$$

R, in this case, has the value $8{\cdot}314 \times 10^7$ ergs per degree.

Thus,
$$M = \frac{8{\cdot}314 \times 10^7 \times 293 \times 4{\cdot}6 \times 10^{-13}}{5{\cdot}96 \times 10^{-7} \times (1 - 0{\cdot}736 \times 0{\cdot}998)}$$
$$= \underline{70{,}850.}$$

(*c*) DIFFUSION

When a system is at equilibrium, the distribution of any molecular species is uniform throughout any single phase. Thus, if a protein solution is placed in contact with pure solvent, the protein molecules diffuse from the region of high concentration into the solvent until equilibrium is achieved. This molecular movement or diffusion stems from the thermal energy of the molecules. The speed with which a molecule moves is characterized by its *diffusion coefficient* (or *constant*) which is a function of the size and shape of the molecule. Knowledge of the diffusion coefficient enables the molecular weight to be determined; it is also necessary for ultracentrifuge studies and affords information as to the shape of molecules in solution.

Two types of diffusion may be recognized—translational and rotational. For the former, free diffusion is permitted to occur, or it may be modified by the application of an external force (such as centrifugal force in the sedimentation equilibrium technique). Rotational diffusion is characteristic of the type of molecule. A spherical molecule is characterized by a single rotary diffusion coefficient, an ellipsoid of revolution by two coefficients, one for each axis, and a general ellipsoid molecule by three coefficients, one corresponding to each of the axes. Rotary diffusion coefficients are determined by flow birefringence and, in the case of molecules that are dipolar, electrical methods.

A little explanation is, perhaps, advisable at this point on the subject of ellipsoids. Ellipsoids of revolution are termed prolate if they are elongated and oblate if flattened. This is made clear in Fig. 1.3, where it will be noticed that the semi-axis of revolution a is greater than the equatorial semi-axis b for a prolate ellipsoid but less than b in the case of an oblate one. The axial ratio a/b is

usually employed to describe these ellipsoids. In most work asymmetric molecules are assumed to be ellipsoids of revolution on account of the complexity of treatment involved if they are considered as general ellipsoids.

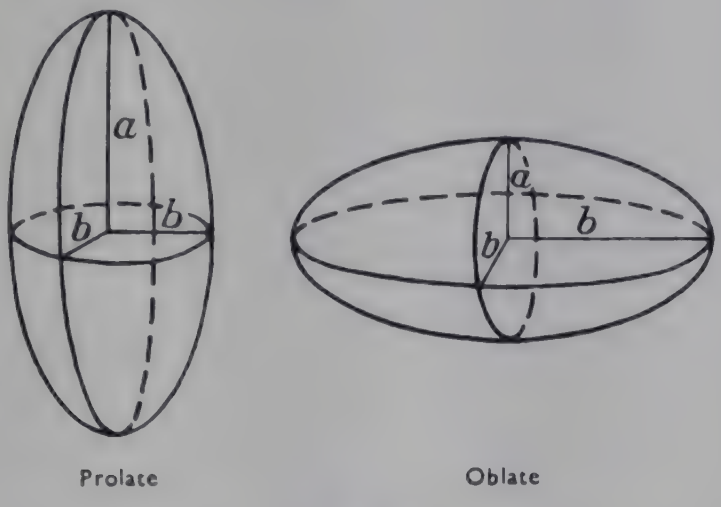

FIG. 1.3
Ellipsoids of revolution. (After ALEXANDER & JOHNSON, 1949.)

TRANSLATIONAL DIFFUSION.—Fick, assuming analogy with the conduction of heat, stated that the mass of solute *ds* diffusing in time *dt* across an area *A* is proportional to the concentration gradient *dc*/*dx* at that point and to the area *A*. This is Fick's First Law and is expressed thus :

$$ds = -DA \frac{dc}{dx} dt,$$

where *dc* is the change in solute concentration over distance *dx* and *D* is the diffusion coefficient. *D* is defined as the quantity of material diffusing per second across a surface area of one square centimetre when the concentration gradient is unity. The minus sign indicates that diffusion is in the direction of the lower concentration. For amino acids in water at 20°, *D* is of the order of 10^{-5} cm.^{2}/sec. and for proteins 10^{-6} to 10^{-7} cm.^{2}/sec. With dilute solutions of non-electrolytes, *D* is related to the *molar frictional coefficient F*, which is the force which must act on 1 gram-molecule of the diffusing substance to give it a velocity of 1 cm./sec., in the following way

$$D = \frac{RT}{F} \quad . \quad . \quad . \quad . \quad (1.10)$$

The molar frictional coefficient $F = Nf$, where f is the force that acts *per molecule* and N is the Avogadro number ($6{\cdot}06 \times 10^{23}$). For spherical molecules of radius r Stokes' Law, which shows the relationship to the viscosity η of the medium, may be applied.

$$f = 6\pi\eta r \quad . \quad . \quad . \quad . \quad (1.11)$$

Thus,
$$D = \frac{RT}{F} = \frac{RT}{Nf} = \frac{RT}{6\pi\eta r N} \quad . \quad . \quad (1.12)$$

Since the volume of a sphere of radius r is $\frac{4}{3}\pi r^3$ and the volume of a spherical molecule is $\frac{M\bar{v}}{N}$, where $\bar{v}$ is the partial specific volume and M the molecular weight, it follows that

$$\bar{v} = \frac{4\pi r^3 N}{3M} \quad . \quad . \quad . \quad (1.13)$$

and
$$r = \left(\frac{3M\bar{v}}{4\pi N}\right)^{\frac{1}{3}} \quad . \quad . \quad . \quad (1.14)$$

Hence the theoretical diffusion coefficient for a spherical molecule D_0 is given by the relationship

$$D_0 = \frac{RT}{Nf_0} = \frac{RT}{6\pi\eta N\left(\frac{3M\bar{v}}{4\pi N}\right)^{\frac{1}{3}}} \quad . \quad . \quad (1.15)$$

and this enables M to be evaluated. Furthermore, the frictional force per spherical molecule f_0 may be expressed by:

$$f_0 = 6\pi\eta\left(\frac{3M\bar{v}}{4\pi N}\right)^{\frac{1}{3}} \quad . \quad . \quad . \quad (1.16)$$

Experimentally determined values of D are usually smaller than those calculated from molecular volumes on the assumption that the proteins are spherically shaped. The ratio D_0/D, which is equal to f/f_0, is a measure of the extent to which the protein molecule deviates in shape from a perfect sphere. This ratio is known as the *dissymmetry constant* or *frictional ratio* and is usually assumed to be related to the ratio of the major and minor axes of an ellipsoid of revolution. Of the proteins investigated at the present time, ribonuclease most closely approaches a spherical

molecule with a frictional ratio of 1·04, whereas tobacco mosaic virus, with a ratio of 3·12, is a "cigar-shaped" molecule.

The study of the shape of protein molecules is complicated by the contribution of hydration which exerts its greatest effect on D_0 values for spherical molecules, the evaluation of which is usually based on the assumption that the protein is anhydrous. Hydration causes a swelling of the molecule by binding water, and this increases the frictional effect. The hydration contribution becomes negligible, however, when the frictional ratio is greater than 2·5.

There are two main methods available for the measurement of translational diffusion, namely analytical and refractometric. In the former, chemical analysis of two solutions of different solute concentration, separated by a porous disc, is carried out over an interval of time. Usually one starts with a fairly concentrated protein solution on one side of the disc and pure solvent on the other. The diffusion coefficient is then given by the expression

$$D = \frac{sh}{ctA} \quad . \quad . \quad . \quad . \quad (1.17)$$

where s is the amount of material diffusing in time t, c the concentration of the solution, h the effective distance through which the solute diffuses and A the effective area of the pores of the disc. The ratio h/A is a constant for any given disc and is known as the membrane constant. It may be evaluated by calibration with low molecular weight substances of known diffusion coefficient. Furthermore, it is only necessary to know the percentage of quantity of material which has traversed the membrane; knowledge of absolute quantities is not needed. Equation 1.17 can be further simplified if the amount of solute contained in 1 ml. of the concentrated solution is taken as unity and the amount which has diffused is expressed in this same unit (i.e. as the number of ml. of the concentrated solution containing the quantity diffused) and becomes

$$D = \frac{Q_{\text{ml}} \cdot h}{tA} \quad . \quad . \quad . \quad . \quad (1.18)$$

where Q_{ml} is the number of millilitres of concentrated solution that contains the amount of substance diffused. For instance, if 0·005 mole of glycerol diffused into water from a 0·1 M solution

Q_{ml} would be 50, since 0·005 mole glycerol is contained in 50 ml. of 0·1 M solution.

In the refractometric method the diffusing boundary is observed by means of a Schlieren cylindrical lens system and is recorded photographically. Other methods are, however, available.

Example 1.8.—Northrop and Anson carried out experiments to determine the diffusion coefficient of haemoglobin at 5° by means of the porous disc method. A 1 per cent. solution of haemoglobin was permitted to diffuse for 2·06 days and the amounts of haemoglobin passing through the disc were estimated colorimetrically at intervals of time. The membrane constant was evaluated by the use of hydrochloric acid, lactose and several salts ; the same value of 0·150 was obtained in each case.

Results from one such experiment were as follows:

Time (days)	0·75	0·92	2·06
Haemoglobin diffused (ml. concentrated solution)	0·202	0·264	0·558

Calculate the average diffusion coefficient.

(Data from NORTHROP & ANSON (1929), *J. gen. Physiol.*, **12**, 543.)

$$D = \frac{Q_{ml}}{t} \cdot \frac{h}{A} \qquad \text{and } \frac{h}{A} = 0{\cdot}150$$

(i) $$D = \frac{0{\cdot}202 \times 0{\cdot}150}{0{\cdot}75} = 0{\cdot}0405$$

(ii) $$\frac{0{\cdot}264 \times 0{\cdot}150}{0{\cdot}92} = 0{\cdot}0430$$

(iii) $$\frac{0{\cdot}558 \times 0{\cdot}150}{2{\cdot}06} = 0{\cdot}0407$$

Hence the average diffusion coefficient is 0·0414 cm.^{2} per day.

Example 1.9.—The diffusion coefficient of a 2·5 per cent. solution of haemoglobin in 0·05 *M* phosphate buffer pH 6·8 and at 5° had an average value of 0·0420 cm.^{2} per day, according to Northrop and Anson. The viscosity coefficient of water at 5° is 0·01519 erg seconds per cm.^{3} and the partial specific volume of dry haemoglobin is 0·75. Taking the molecular weight of haemoglobin to be 67,000 calculate the degree of hydration of the protein.

(Data from KUNITZ, ANSON & NORTHROP (1934), *J. gen. Physiol.*, **17**, 365.)

The volume of hydrated molecules in solution is, per mole, $\frac{4}{3}\pi r^3 N$ and the volume of one mole of dry protein $M\bar{v} = 67000 \times 0{\cdot}75 = 50{,}260$. The volume of water of hydration per mole will be given by

$$\frac{4}{3}\pi r^3 N - M\bar{v}.$$

The radius of a hydrated molecule r may be obtained from the diffusion coefficient by means of equation 1.12.

$$r = \frac{RT}{6\pi\eta N D}.$$

Care must be exercised in the units employed for the diffusion coefficient ; it

is given as cm.^{2} per day which must be converted to cm.^{2} per second before substitution in the above equation, i.e. $0{\cdot}0420/60 \times 60 \times 24$

$$r = \frac{8{\cdot}314 \times 10^7 \times 278 \times 60 \times 60 \times 24}{6 \times 3{\cdot}142 \times 0{\cdot}01519 \times 6{\cdot}06 \times 10^{23} \times 0{\cdot}0420}$$
$$= 2{\cdot}74 \times 10^{-7} \text{ cm.}$$

This value for r can now be substituted in the first equation and the volume of water of hydration per mole will be

$$\frac{4 \times 3{\cdot}142 \times (2{\cdot}74 \times 10^{-7})^3 \times 6{\cdot}06 \times 10^{23}}{3} - 50{,}260 \text{ cm.}^3$$
$$= 52210 - 50260 = \underline{1{,}950 \text{ cm}^3.}$$

And the water of hydration per gram dry protein $= \frac{1950}{67000} = \underline{0{\cdot}029 \text{ cm.}^3.}$

(*d*) Viscosity

Viscosity affords a secondary method for determining molecular weights and depends for its success upon calibration against primary methods of the types previously discussed. The approach used is essentially of an empirical nature.

Viscosity is usually determined by one of two methods.

1. The Capillary Flow Method.—The rate of flow of liquid through a capillary tube of known radius and length and under a known pressure is measured.

2. The Couette or Concentric Flow Method.—This consists of two coaxial cylinders with liquid between them; the outer cylinder is rotated at constant speed, and the inner, suspended freely by a torsion wire, has a turning moment exerted on it, the angle through which it is deflected being measured by means of a light beam, mirror and scale.

The Ostwald viscometer is an example of the capillary flow method where the liquid flows under its own head of pressure. For these conditions

$$\eta = C\rho t \quad . \quad . \quad . \quad . \quad (1.19)$$

where η is the viscosity coefficient, C a constant depending upon the dimensions of the capillary, ρ the density of the liquid and t the time of flow of the liquid between two fixed points on the viscometer. If the times are compared for the flow of a protein solution and for water (viscosity coefficients η and η_0 respectively), then, since the density of water ρ_0 is unity, the *relative viscosity* η_r is given by the expression

$$\eta_r = \frac{\eta}{\eta_0} = \frac{C\rho t}{C\rho_0 t_0} = \frac{\rho t}{t_0} \quad . \quad . \quad . \quad (1.20)$$

The *specific viscosity* η_{sp} is defined as

$$\eta_{sp} = \frac{\eta}{\eta_0} - 1 = \eta_r - 1 \quad . \quad . \quad . \quad (1.21)$$

For spherical particles and very dilute solutions Einstein formulated the equation

$$\frac{\eta}{\eta_0} = \eta_r = \frac{1 + 0{\cdot}5\phi}{(1 - \phi)^2} \quad . \quad . \quad . \quad (1.22)$$

which can be expanded into a power series, only the first term of which is significant, and equation 1.22 thus becomes

$$\eta_r = 1 + 2{\cdot}5\phi \quad . \quad . \quad . \quad (1.23)$$

where ϕ is the volume fraction of the solute, i.e. the volume occupied by solute molecules relative to the volume of the system. Thus, if n molecules of volume v are present in a total volume of solution V, the volume fraction $\phi = nv/V$, and if n is known it is possible to calculate the volume occupied by each particle or molecule and hence its degree of hydration.

Equation 1.23 may be written in the form

$$\frac{\eta_r - 1}{\phi} = \frac{\eta_{sp}}{\phi} = 2{\cdot}5 \quad . \quad . \quad . \quad (1.24)$$

so that a plot of η_{sp}/ϕ against ϕ should give a straight line parallel to the ϕ axis and cutting the η_{sp}/ϕ axis with an intercept of 2·5. This affords a method of calculating the hydration of spherical molecules. The intercept should be 2·5, and therefore the additional volume (equal to the hydration) which must be added to the volume of the suspended particles in the dry state to obtain this intercept can be calculated. Equation 1.24 indicates that the relative viscosity at low concentration is independent of both particle size and the nature of the solute, due to the fact that the increased viscosity is caused by indirect action of the particles on solvent flow. Particle size cannot therefore be obtained by use of this equation.

For asymmetric molecules the Einstein equation (1.22, 23) does not hold, and many workers have introduced empirical equations to fit better the observed data. Kunitz proposed the expression

$$\eta_r = \frac{1 + 0{\cdot}5\phi}{(1 - \phi)^4} \quad . \quad . \quad . \quad (1.25)$$

which, when expanded, approximates to

$$\eta_r = 1 + 4{\cdot}5\phi \quad . \quad . \quad . \quad (1.26)$$

This approximation proves satisfactory for small values of ϕ. A strict hydrodynamic derivation for hydrated spherical molecules where f/f_0 departs from unity is the following, due to Polson

$$D = \frac{9{\cdot}35 \times 10^{-6}}{M^{\frac{1}{3}}\eta_{sp}^{\frac{1}{3}}} \quad . \quad . \quad . \quad (1.27)$$

Staudinger studied the viscosity of long chain polymers and related viscosity to molecular weight by the equation

$$\frac{\eta_{sp}}{c} = K_m M \quad . \quad . \quad . \quad . \quad (1.28)$$

where K_m is a constant characteristic of each polymer system and c the concentration, usually in grams of solute per 100 ml. solution.

Now the weight *intrinsic viscosity* $[\eta]$ is defined as

$$\lim_{c \to 0} \left(\frac{\eta_{sp}}{c}\right) = [\eta] \quad . \quad . \quad . \quad (1.29)$$

and can be obtained by plotting η_{sp}/c against c and extrapolating to zero concentration. Therefore, on account of the variation of η_{sp}/c with c, equation 1.28 should be replaced by

$$\lim_{c \to 0} \left(\frac{\eta_{sp}}{c}\right) = [\eta] = K_m M \quad . \quad . \quad (1.30)$$

In determining $[\eta]$ for molecular weight calculations it is customary to measure the viscosities of several solutions whose concentrations are not greater than 1 gram per 100 ml. solution and then to plot the values as described above in order to get the value of $[\eta]$ at zero concentration.

The volume intrinsic viscosity is given by $\lim_{\phi \to 0} \left(\frac{\eta_{sp}}{\phi}\right)$ and for unhydrated spheres is 2·5.

Example 1.10.—The relative viscosity of lactose solutions of varying concentration was measured by Kunitz at 25° with the following results:

Lactose concentration g./100 ml.	2·8	5·9	12·1	18·3	25·7
Relative viscosity η_r	1·086	1·187	1·450	1·779	2·371

What is the effect of lactose concentration on the specific volume fraction? Use the empirical equation derived by Kunitz.

(Data from KUNITZ (1926), *J. gen. Physiol.*, **9**, 715.)

The expanded form of the Kunitz equation 1.26 is

$$\eta_r = 1 + 4{\cdot}5\phi,$$

whence $\phi = \dfrac{\eta_r - 1}{4{\cdot}5}$ and can be calculated for each value of η_r. Thus for 2·8 g. lactose/100 ml. $\phi = \dfrac{1{\cdot}086 - 1{\cdot}0}{4{\cdot}5} = 0{\cdot}0191$ and the specific volume fraction $= \dfrac{0{\cdot}0191}{2{\cdot}8} = 0{\cdot}00682$.

Other values are recorded in the following table:

Concentration (c) g/100 ml.	*Volume fraction (ϕ)*	*Specific volume fraction (ϕ/c)*
2·8	0·0191	0·00682
5·9	0·0415	0·00703
12·1	0·1000	0·00826
18·3	0·1664	0·00909
25·7	0·3046	0·0118

From these figures it would appear that the specific volume fraction increases with increase in lactose concentration, indicating that there is a hydration effect. If, however, the full Kunitz equation 1.25 is used instead of the expanded approximation, reasonably constant values of the specific volume fraction are obtained. This is because equation 1.26 holds only for small values of ϕ. Solving a fourth-power equation is not an easy process however and Kunitz prepared a graph which enabled him to read off values of ϕ from the specific viscosity of the solution.

(*e*) OTHER METHODS

Alternative physico-chemical methods of molecular weight determination include flow birefringence, light scattering and dielectric dispersion measurements. For details of these procedures the student is referred to Cohn & Edsall (1943) and Alexander & Johnson (1949).

SUGGESTED READING

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PROBLEMS

1.1. A compound contained 10·66 per cent. sulphur and 15·5 per cent. phosphorus. Determine the minimal molecular weight and the number of sulphur and phosphorus atoms present in this molecular weight.

1.2. The following analytical data, in percentages, are available for horse haemoglobin: iron, 0·335; disulphide sulphur, 0·190; sulphur, 0·390. For pig haemoglobin the iron and sulphur content is respectively 0·40 and 0·48 per cent. Compare the minimal molecular weights of the haemoglobins from these two species and deduce the number of atoms of these elements present in the molecule.

(After COHN, HENDRY & PRENTISS (1925), *J. biol. Chem.*, **63**, 721.)

1.3. Amino acid analysis of the wheat protein glutenin has given the following percentage composition for three amino acids present in small amounts: tryptophan, 1·68; tyrosine, 4·5; β-hydroxyglutamic acid, 1·8.
Use this information to calculate the minimal molecular weight and deduce the number of molecules of each amino acid present in the protein molecule.

(After COHN, HENDRY & PRENTISS (1925), *J. biol. Chem.*, **63**, 721.)

1.4. The copper content of haemocyanins isolated from various species has been determined. The figures given are the percentage of copper present: Cancer, 0·32; Homarus, 0·34; Octopus vulgaris, 0·38; Watferit, 0·29; Limulus polyphemus, 0·173. Compare the minimal molecular weights of these haemocyanins of different origin.

(After REDFIELD, COOLIDGE & SHOTTS (1928), *J. biol. Chem.*, **76**, 185.)

1.5. Analysis of ox haemoglobin gave the following percentages: iron, 0·336; sulphur, 0·48; arginine content, 4·24. Calculate the minimal molecular weight and the number of these atoms or molecules assumed to be present.

(After COHN, HENDRY & PRENTISS (1925), *J. biol. Chem.*, **63**, 721.)

1.6. Hüfner determined experimentally that one gram of haemoglobin combines with a maximum of 1·34 ml. of oxygen at N.T.P. Subsequently it was shown by Cohn and co-workers that horse haemoglobin has an iron content of 0·335 per cent. Use these data to deduce the stoichiometric relationship between oxygen combination and iron content for haemoglobin.

(After HÜFNER (1894), *Arch. Anat. Physiol.*, 130; and COHN, HENDRY & PRENTISS (1925), *J. biol. Chem.*, **63**, 721.)

1.7. The maximal acid- and base-combining capacities per gram for myogen are $1{\cdot}35 \times 10^{-3}$ and $1{\cdot}28 \times 10^{-3}$ equivalents respectively. Compare the minimal molecular weights obtained by these two titrations.

1.8. Gelatin has a maximal acid-combining capacity of 96×10^{-5} equivalents per gram, according to Hitchcock. Determine the minimal molecular weight and compare this figure with the one obtained from Simms' value of 7×10^{-4} equivalents base combining per gram gelatin.

(Data from HITCHCOCK (1931), *J. gen. Physiol.*, **15**, 125; and SIMMS (1928), *J. gen. Physiol.*, **11**, 629.)

1.9. Serum albumin has been reported to possess maximal acid- and base-combining capacities of 72 and 70×10^{-5} equivalents per gram respectively. If the figure of 67,100 for the molecular weight, obtained by diffusion and sedimentation velocity measurements, is accepted, calculate the approximate number of acidic and basic groups per serum albumin molecule.

(Data from PRIDEAUX & WOODS (1932), *Proc. roy. Soc.*, B, **111**, 201; and LAMM & POLSON (1936), *Biochem. J.*, **30**, 528.)

1.10. Edestin has a maximal acid-combining capacity of 134×10^{-5} equivalents per gram and the molecular weight obtained by sedimentation measurements is 309,000. Ascertain the approximate number of basic groups in the edestin molecule.

(Data from HITCHCOCK (1930), *J. gen. Physiol.*, **14**, 99; and POLSON (1939), *Kolloid Z.*, **87**, 149.)

1.11. Fibrin was titrated with an acidic dye and gave a combining capacity of $1{\cdot}475 \times 10^{-3}$ equivalents per gram protein. Calculate the equivalent combining weight of the protein cation.

(Data from CHAPMAN, GREENBERG & SCHMIDT (1927), *J. biol. Chem.*, **72**, 707.)

1.12. Gelatin has been titrated with acid, base, acidic and basic dyes. The combining capacities in equivalents per gram protein were as follows :

Acid:	$9{\cdot}6 \times 10^{-4}$	Acidic dye:	$1{\cdot}04 \times 10^{-3}$
Base:	$7{\cdot}0 \times 10^{-4}$	Basic dye:	$7{\cdot}0 \times 10^{-4}$

Compare the equivalent combining weights of the protein cation and anion and comment on the agreement between acid-base and dye titrations.

(Data from PRIDEAUX & WOODS (1932), *Proc. roy. Soc.*, B, **110**, 353; RAWLINS & SCHMIDT (1929), *J. biol. Chem.*, **82**, 709; and CHAPMAN, GREENBERG & SCHMIDT (1927), *J. biol. Chem.*, **72**, 707.)

1.13. The composition of a macromolecule corresponds to the following:

Number of Moles	*Molecular Weight*
10	17,000
10	34,000
20	68,000

Calculate the number-average and the weight-average molecular weights.

1.14. Calculate the osmotic pressure of a 1 per cent. (w/v) solution of glycerol at 22° C.

1.15. A solution containing 0·5 gram of urea per 100 ml. exerted an osmotic pressure of 2·037 atmospheres at 25° C. Determine the molecular weight of urea.

1.16. The following figures were obtained in an investigation of the osmotic pressure of a high molecular weight substance.

Concentration g./100 ml.	2·0	2·75	3·4	5·0	6·0	7·25	8·3
Osmotic pressure mm. Hg	6·6	9·1	11·6	17·9	21·9	27·4	32·8

By use of a graphical method, determine the molecular weight of the compound.

1.17. Pfeffer obtained the following results with dilute solutions of sucrose at 0° C.

Concentration of sucrose (g. per litre solution)	10·03	20·14	40·60	61·38
Osmotic pressure (atmospheres)	0·686	1·34	2·75	4·04

Use these figures to demonstrate that the osmotic pressure of a solution at constant temperature is proportional to the concentration.

The relationship between the osmotic pressure and absolute temperature of a 1 per cent. solution of sucrose was investigated with the following results.

Absolute temperature	273·0	279·8	286·7	287·2	288·5	295·0	305·0
Osmotic pressure (atmospheres)	0·648	0·664	0·691	0·671	0·684	0·721	0·716

Deduce the relationship between osmotic pressure and absolute temperature from these data.

(After PFEFFER (1877), *Osmotische Untersuchungen*, Leipzig.)

1.18. What special principles are involved when osmometry is applied to high molecular weight charged particles such as proteins? Two different proteins A and B give the following osmotic data at 5° C.:

Concentration (g./litre)		10	20	30	40
Osmotic pressure in atmospheres	A	0·0038	0·0078	0·0120	0·0160
	B	0·0075	0·0220	0·0423	0·0720

Comment on the data and calculate molecular weights.
(R = 0·08207 litre atmospheres/mole/°K)

(Leeds Honours Course Finals, 1953.)

1.19. Berkeley and Hartley determined the osmotic pressure of sucrose solutions at 0° C. with the following results:

Concentration (g./litre)	2·02	10·0	20·0	35·0	93·75
Osmotic pressure (atmospheres)	0·134	0·66	1·32	2·97	6·18

Show that these findings are in accordance with Boyle's Law as applied to dilute solutions.

(Data from BERKELEY & HARTLEY (1906), *Phil. Trans. roy. Soc.*, **206**, A, 481.)

1.20. The following osmotic pressure data were obtained for serum albumin in 0·05 M acetate buffer at the isoelectric point pH 4·8 and 0°.

Concentration (g./100 ml. solvent)	0·78	1·25	2·79	2·82	3·38	4·18	8·98	12·45
Observed osmotic pressure (cm. water)	2·39	4·01	8·53	8·68	10·80	13·01	27·84	37·69

Use these data to determine the molecular weight of serum albumin.

(After BURK (1932), *J. biol. Chem.*, **98**, 353.)

1.21. The effect of hydrogen ion concentration on the osmotic pressure of dialysed sheep haemoglobin was investigated by Adair. Solutions of varying haemoglobin concentration were used and the experimental temperature was 23°. Below, the osmotic pressure is expressed in terms of the pressure in mm. of mercury per 1 per cent. protein at 0°.

pH	5·0	5·4	6·5	6·7	6·8	6·8	6·8	7·2	9·6	10·2
Osmotic pressure mm. Hg	21·5	13·4	3·2	2·4	2·4	3·5	4·5	5·0	15·6	21·4

Express these data graphically, comment on the results obtained and deduce the isoelectric point of the haemoglobin.

(After ADAIR (1925), *Proc. roy. Soc.*, A, **109**, 292.)

1.22. The osmotic pressure of solutions of serum albumin in 0·0667 M phosphate buffer pH 7·4 and 0° has been determined and the results are tabulated below.

Concentration (g./100 ml. solvent)	0·578	0·684	1·05	1·59	2·27	2·47	2·95
Osmotic pressure (cm. H_2O)	2·04	2·35	3·56	5·62	8·55	9·88	10·84

Determine the molecular weight of serum albumin.

(After ROCHE & MARQUET (1935), *C.R. soc. biol.*, **118**, 898.)

1.23. The osmotic pressure of carbon monoxide haemoglobin prepared from horse blood was measured at various concentrations at pH 7·38 and 0° C. The results obtained are tabulated below.

Concentration of HbCO g./100 ml.	3·028	3·015	2·140	2·025	2·010	0·976	0·978
Osmotic pressure mm. Hg	8·50	8·43	5·81	5·54	5·46	2·55	2·59

Determine the molecular weight of haemoglobin from these measurements.

(Data from ADAIR (1949), in *Haemoglobin*, p. 191, ed. Roughton & Kendrew. London: Butterworth.)

1.24. The osmotic pressure of human haemoglobin in 0·2 M phosphate has been measured by Gutfreund. The following table presents his data obtained at 3°.

Concentration g./100 ml.	*Osmotic pressure cm.* H_2O
0·47	1·6
0·56	2·0
0·60	2·2
1·29	4·7
1·66	6·5
2·39	9·5
3·09	11·3
3·25	12·8
3·47	13·2
3·75	14·9
4·34	17·6
4·83	19·9
5·40	22·6
7·01	30·6

Use these figures to calculate the molecular weight of human haemoglobin.

(Data from GUTFREUND (1949), in *Haemoglobin*, p. 197, ed. Roughton & Kendrew. London: Butterworth.)

1.25. The action of ultraviolet light on cellulose nitrate has been studied by measuring the osmotic pressure of the solution of cellulose nitrate in acetone. The solution, containing 10·28 g. of cellulose nitrate per litre, was exposed to ultraviolet irradiation for 10·2 days and osmotic measurements were made by a dynamic method after 2·08 days and at the end of the experiment. The pressure on the solution was altered and the rate of movement of a meniscus, indicating

the movement of solvent into or out of the solution, measured. Data obtained are recorded below. Direction of meniscus movement is indicated by + and −.

Time of exposure (days)	*Temperature ° C.*	*Pressure cm. acetone*	*Rate of movement of meniscus cm. per sec.*
2·08	25	46	+0·0048
		56	+0·0067
		67	+0·0096
		77	+0·0116
10·2	22	36	−0·0168
		46	−0·0068
		67	+0·0108
		77	+0·0200

From these figures ascertain the effect of ultraviolet irradiation on the molecular weight of cellulose nitrate. The density of acetone is 0·78 and that of mercury 13·59.

(After MONTONNA & JILK (1941), *J. phys. Chem.*, **45**, 1374.)

1.26. The molecular weight of the dye congo red has been determined by the sedimentation equilibrium method. The initial concentration of the dye was 0·1 g. per litre dissolved in 0·1 M NaCl, the density of the resulting solution being 1·0023. The speed of rotation of the centrifuge was 299·6 revolutions per second. The following data were obtained at 20°.

Distance from axis of rotation (cm.)		*Relative concentration of congo red*	
x_2	x_1	c_2	c_1
5·87	5·84	53·60	50·46
5·81	5·78	47·57	44·79
5·75	5·72	42·18	39·76
5·66	5·63	35·36	33·36

The partial specific volume of congo red is 0·60. Calculate the mean molecular weight from these figures.

(Data from SVEDBERG & PEDERSEN (1940), *The Ultracentrifuge.* Oxford University Press.)

1.27. The sedimentation equilibrium technique has been used to determine the molecular weight of the erythrocruorin of *Planorbis*. The experiment was carried out at 20° and the density of the solvent was 1·0034. The centrifuge speed was 2280 r.p.m. and the distance of the outer end of the solution from the axis of rotation was 5·95 cm. Experiments were conducted for 48, 53 and 77 hours and below are tabulated some of the experimental findings.

Distance from axis of rotation (cm.)		*Concentration ratio*
x_1	x_2	c_2/c_1
5·85	5·80	1·289
5·70	5·65	1·195
5·60	5·55	1·237
5·55	5·50	1·223

The partial specific volume of erythrocruorin was found to be 0·745. Determine the mean molecular weight.

(After SVEDBERG & ERIKSSON-QUENSEL (1934), *J. Amer. chem. Soc.*, **56**, 1700.)

1.28. The effect of pepsin on a diphtheria antitoxic pseudoglobulin from the horse has been examined by means of the ultracentrifuge. The sedimentation constants before and after pepsin treatment were 7·2 and 5·7 $\times$ 10^{-13} sec. respectively and the corresponding diffusion constants were 3·9 and 5·8 $\times$ 10^{-7} cm.^{2}/sec. (all values corrected to values for water at 20°). The partial specific volume of the protein was assumed to be the same as normal horse globulin and to have a value of 0·745. The density of water at 20° is 0·9982. Determine the effect of the pepsin treatment on the molecular weight of the antitoxin.

(After PETERMANN & PAPPENHEIMER, Jr. (1941), *J. phys. Chem.*, **45**, 1.)

1.29. The maximum molecular weight of insulin has been determined by sedimentation and diffusion studies. The sedimentation constant, corrected to the value in water at 20°, is 3·12 $\times$ 10^{-13} sec. and the diffusion coefficient, similarly corrected, is 8·2 $\times$ 10^{-7} cm.^{2}/sec. The partial specific volume of insulin was found to be 0·735, and the density of water at 20° is 0·9982. Evaluate the molecular weight.

(After CREETH (1953), *Biochem. J.*, **53**, 41.)

1.30. Studies on the sedimentation and diffusion of human albumins yielded the following constants (corrected to their values in water at 20°): s_{20} 4·24 $\times$ 10^{-13} sec. and D_{20} 6·32 $\times$ 10^{-7} cm.^{2} sec.$^{-1}$. The density of water at 20° is 0·9982 and the partial specific volume of the albumins was taken as 0·733. Calculate the molecular weight.

(After CHARLWOOD (1952), *Biochem. J.*, **51**, 113.)

1.31. The Lewis blood-group substance was found to have the following characteristics. At a concentration of 1g./100 ml. the sedimentation constant was 5·44 $\times$ 10^{-13} sec. and the diffusion coefficient 1·37 $\times$ 10^{-7} cm.^{2}/sec., both values being corrected to values in water at 20°. The partial specific volume was found to be 0·643. The density of water at 20° is 0·9982. Calculate the molecular weight of this substance.

(After KEKWICK (1952), *Biochem. J.*, **50**, 471.)

1.32. 20 g. crystalline trypsin were dissolved in 0·25 saturated $(NH_4)_2SO_4$ acetate buffer pH 4·0 and placed in a rocking osmometer in the same solvent at 5°. The osmotic pressure was determined after 24 hours and the solution analysed for protein nitrogen.

Protein concentration mg./ml.	72	71	50	49	22·5	18
Pressure mm. Hg	39	38	22	21·5	11	9

The diffusion coefficient of trypsin was found to be 0·020 cm.^{2}/day and the viscosity coefficient of the solvent was 0·0303 erg sec./cm.^{3} at 5°. Determine the molecular weight of trypsin and the average molar volume of the hydrated protein.

(After NORTHROP & KUNITZ (1933), *J. gen. Physiol.*, **16**, 295.)

1.33. In an experiment to determine the diffusion coefficient of crystalline trypsin, the following results were obtained by the porous-disc method. Diffusion was followed by both trypsin activity measurements and by determination of nitrogen.

Original solution mg. N/ml.	*Time days*	*Quantity of original solution diffused by activity ml.*	*Quantity of original solution diffused by nitrogen content ml.*
3·45	0·156	0·062	——
	0·708	0·281	0·274
	2·00	0·565	0·806

The solvent (0·5 saturated $MgSO_4$, 0·1 M acetate) had a specific gravity of 1·115 and its viscosity coefficient was 0·0303 erg sec./cm.^{3}. All measurements were carried out at 5° and the membrane constant had been evaluated as 0·054.

Calculate the average diffusion coefficient in cm.^{2}/day and compare the values obtained by activity measurements with those obtained by nitrogen determinations.

What is the average radius of the hydrated trypsin molecule in solution? Determine the average molar volume of the protein.

(After SCHERP (1933), *J. gen. Physiol.*, **16**, 795.)

1.34. The viscosity of trypsin solutions of various concentrations was measured by means of an Ostwald viscometer. The protein was in 0·5 saturated magnesium sulphate and 0·1 M acetate buffer pH 4·0, and at 5° the following results were obtained.

Trypsin concentration g./100 ml.	0	0·8	1·6	2·4	3·2	4·0
Time of outflow sec.	203·4	212·6	221·5	231·0	241·4	257·0

Neglecting any changes in the density of the trypsin solutions, determine the effect of concentration on the degree of hydration of trypsin. The partial specific volume of trypsin may be taken as 0·75. Use the Kunitz equation $\eta_r = 1 + 4{\cdot}5\phi$ to evaluate the volume fraction.

(After KUNITZ, ANSON & NORTHROP (1934), *J. gen. Physiol.*, **17**, 365.)

1.35. The van't Hoff equation for osmotic pressure does not hold for solutions that are not dilute, neither for solutes that are hydrated in solution. A suggested correction for the equation is the following, where the volume fraction ϕ represents the actual volume occupied by the hydrated molecules

$$\Pi = \frac{RT}{M} \frac{c}{(1000-\phi)}$$

and where c is the concentration in grams per litre.

Compare the average value for the molecular weight of gelatin obtained by the use of the above equation with that obtained graphically by the plot of the reduced osmotic pressure as a function of the gelatin concentration. Use the Kunitz equation to evaluate the volume fraction. Temperature 35°.

Gelatin concentration g./100 ml.	*Relative viscosity*	*Osmotic pressure mm. Hg*
1	1·43	3·5
2	2·06	7·5
3	2·96	12·0
4	4·24	17·0
5	6·00	23·0
6	8·20	29·5

(After KUNITZ, (1927) *J. gen. Physiol.*, **10**, 811.)

1.36. Kunitz proposed the following empirical equation to replace the Einstein viscosity equation for spheres

$$\eta_r = \frac{1 + 0{\cdot}5\phi}{(1 - \phi)^4}.$$

This equation can be expanded and approximates to $\eta_r = 1 + 4{\cdot}5\phi$. Compare

the volume fractions calculated by the Kunitz and Einstein equations from the following data, obtained with saccharose and glucose.

Saccharose g./100 ml.	1·0	2·0	4·9	10·3	15·6	21·7
Relative viscosity η_r	1·026	1·054	1·141	1·329	1·570	1·917
Glucose g./100 ml.	2·1	4·7	10·6	16·6	21·7	26·4
Relative viscosity η_r	1·062	1·130	1·316	1·619	1·901	2·216

(Data from PULVERMACHER (1920), *Z. anorg. allg. Chem.*, **113**, 147, quoted by Kunitz (1926), *J. gen. Physiol.*, **9**, 715.)

1.37. The viscosity of carbon monoxide haemoglobin was investigated at pH 6·8 and 5°, when the following data were obtained:

Protein concentration g./100 ml.	2·10	4·20	6·30	8·36	10·45
Relative viscosity η_r	1·084	1·175	1·290	1·445	1·610

The partial specific volume of dry haemoglobin is 0·75. Determine the volume of water of hydration per gram haemoglobin in order to ascertain the relationship between hydration and protein concentration. Use the Kunitz equation $\eta_r = 1 + 4{\cdot}5\phi$ to evaluate the volume fraction.

(After KUNITZ, ANSON and NORTHROP (1934), *J. gen. Physiol.*, **17**, 365.)

1.38. The following values of the diffusion coefficient for glucose at a series of concentrations were obtained at 25°:

Concentration (moles/litre)	0·100	0·200	0·250	0·300	0·355	0·400	0·500	0·600
Diffusion coefficient ($cm.^2$/day)	0·566	0·556	0·552	0·550	0·546	0·543	0·539	0·535

Use these data to obtain a value for the diffusion coefficient at infinite dilution and hence calculate the molecular weight of glucose. A linear graph is obtained if the diffusion coefficient is plotted against the square root of the concentration. The viscosity of water at 25° is 0·008937 poise and the density of glucose may be taken as 1·548.

(After FRIEDMAN & CARPENTER (1939), *J. Amer. chem. Soc.*, **61**, 1745.)

1.39. The following data were obtained for crystalline horse-liver alcohol dehydrogenase:

The sedimentation constant was found in two runs to be $S_{20} = 4{\cdot}86$ and 4·90 Svedberg units, and the diffusion coefficient, D_{20}, was found to be $6{\cdot}5 \times 10^{-7}$ cm^2. sec^{-1}. A determination of the partial specific volume gave a value of 0·751. The density of the solution was 0·998.

Use these data to calculate the molecular weight of the enzyme.

(After THEORELL & BONNICHSEN (1951), *Acta chem. scand.*, **5**, 1105.)

CHAPTER II

ACID-BASE RELATIONSHIPS AND ELECTROLYTE BEHAVIOUR OF AMINO ACIDS AND PROTEINS

Acid-base Equilibria.

Brönsted (1923) defined an acid as a compound which donates protons and a base as one which accepts them. Thus

$$\text{acid} \rightleftharpoons \text{base} + H^+ \quad . \quad . \quad . \quad (2.1)$$

Accordingly each acid has its corresponding or conjugate base and acids and bases can react by *proton exchange*, represented by the general equation

$$\text{acid}_1 + \text{base}_2 \rightleftharpoons \text{base}_1 + \text{acid}_2 \quad . \quad . \quad (2.2)$$

or $$a_1 + b_2 \rightleftharpoons b_1 + a_2.$$

An *ampholyte* is a compound which displays both acid and base properties, e.g. water and amino acids

$$2\,H_2O \rightleftharpoons H_3O^+ + OH'$$

$$2\,RCH\overset{+}{N}H_3.COO' \rightleftharpoons RCH\overset{+}{N}H_3.COOH + RCHNH_2.COO'.$$

Although, as will be seen from the above equations, the proton is hydrated in aqueous solution, the hydrogen ion is nevertheless frequently referred to as H^+; both methods are used in this chapter. The hydrogen ion activity (H^+) is usually expressed in terms of its negative logarithm, pH

$$pH = -\log (H^+) \quad . \quad . \quad . \quad (2.3)$$

where (H^+) is the activity. At very great dilution the hydrogen ion activity is equivalent to the *hydrogen ion concentration* [H^+]. This point needs, perhaps, explanation. Only weak electrolytes behave ideally with respect to the colligative properties of their solutions (Chapter I). This is because interaction occurs between charged ions in solution; each ion does not exist as a separate entity but is surrounded by ions of opposite charge. The magnitude of the interaction is determined by the ionic charge and the

concentration and charges of all ionic species present in the solution. Debye and Hückel worked out the theory of this departure from ideal behaviour. Consequently physical chemists have introduced the concept of *activity*. The concentration C_i of a given ion multiplied by its *activity coefficient* f_i gives the activity a_i of that ion, the activity exhibiting ideal behaviour. Thus for hydrogen ions

$$a_{H^+} = f_{H^+}C_{H^+} \text{ or } f_{H^+}[H^+] \quad . \quad . \quad (2.4)$$

At very great dilution the activity coefficient approaches unity and $a_{H^+} = [H^+]$ i.e. $(H^+) = [H^+]$.

Lewis showed that the activity coefficient varies with salt concentration and that the deviation from ideal behaviour of a given kind of ion, provided that the ionic concentration is not too high, depends upon two main factors: (1) the valency of the given ion and (2) the *ionic strength* (I) of the solution. The concept of ionic strength he introduced to enable different electrolytes in similar environments to be compared. It is given by the relationship

$$I = \tfrac{1}{2}\,\Sigma m_i z_i^2 = \tfrac{1}{2}\,\Sigma c z_i \quad . \quad . \quad . \quad (2.5)$$

where m_i is the molarity of the ion, z_i its valency and c its equivalent concentration ($c = m_i z_i$). Thus the ionic strength is half the sum of the products of molar concentration and the square of the valency for each of the ionic species present in solution.

Example 2.1.—Calculate the ionic strength of 0·5 M NaCl and 0·5 M $MgCl_2$ solutions.

0·5 M NaCl $\quad I = \tfrac{1}{2}[0{\cdot}5 \times 1^2 + 0{\cdot}5 \times 1^2]$
$= \underline{0{\cdot}5}$

0·5 M $MgCl_2$ $\quad I = \tfrac{1}{2}[0{\cdot}5 \times 2^2 + 2(0{\cdot}5 \times 1^2)]$
$= \underline{1{\cdot}5}$

In calculating the ionic strength of a weak electrolyte obviously the degree of dissociation must be taken into account to obtain the true ionic concentration.

The activity coefficient varies with salt concentration and for dilute solutions of all electrolytes (e.g. $I < 0{\cdot}1$ M) the activity coefficient of a given ionic species is represented by this approximate equation

$$pf_i = -\log f_i = \frac{Az_i^2\sqrt{I}}{1+\sqrt{I}} \quad . \quad . \quad (2.6)$$

where A is a constant dependent upon the nature and temperature of the solvent (A increases in solvents of low dielectric constant). If the value of A for water is substituted, equation 2.6 for dilute solutions becomes

$$-\log f_i = \frac{0{\cdot}5z_i^2\sqrt{I}}{1+\sqrt{I}} \quad . \quad . \quad . \quad (2.6a)$$

For very dilute solutions ($I<0{\cdot}01$ M) equation 2.6a reduces to

$$-\log f_i = 0{\cdot}5z_i^2\sqrt{I} \quad . \quad . \quad . \quad (2.7)$$

In general terms, for a salt instead of a single ionic species, the *mean activity coefficient* is given by

$$-\log f_{\pm} = 0{\cdot}5z_+z_-\sqrt{I} \; . \quad . \quad . \quad (2.7a)$$

where z_+ and z_- are the valencies (irrespective of sign) of the cation and anion respectively. The Debye-Hückel treatment of more concentrated solutions leads to a modification of equation 2.6, namely

$$-\log f_i = \frac{Az_i^2\sqrt{I}}{1+Bd_i\sqrt{I}} \; . \quad . \quad . \quad (2.6b)$$

where B is a constant and d_i is the ionic diameter. Similarly, equation 2.7a now becomes

$$-\log f_{\pm} = 0{\cdot}5z_+z_-\sqrt{I} - CI \quad . \quad . \quad (2.7b)$$

where C is a constant dependent upon the electrolyte. Thus an electrolyte has the same activity coefficient in all solutions of the same ionic strength.

Example 2.2.—Find the pH value of 0·001 N hydrochloric acid if the acid is completely dissociated.

Here there is no complication regarding the degree of dissociation and the concentration of H^+ ions is 0·001 g. equivalent per litre.

$$\text{Therefore pH} = -\log 0{\cdot}001 = -(\bar{3}{\cdot}000)$$
$$= 3{\cdot}0$$

Example 2.3.—Find the pH value of a 0·001 N solution of acetic acid. Dissociation constant is $1{\cdot}8 \times 10^{-5}$.

Let α be the degree of dissociation and V the dilution, i.e. volume in litres containing 1 gram equivalent of the acid. $V = 1000$ for the 0·001 N solution.

$$CH_3COOH \rightleftharpoons CH_3COO' + H^+$$
$$\frac{1-\alpha}{V} \qquad \frac{\alpha}{V} \qquad \frac{\alpha}{V}$$

Whence $$K_a = \frac{(CH_3COO')(H^+)}{(CH_3COOH)} = \frac{\alpha^2}{V(1-\alpha)}.$$

Since the degree of dissociation is very small we may equate

$$1 - \alpha \text{ to } 1, \qquad \text{i.e. } K_a = \frac{\alpha^2}{V} = \frac{\alpha^2}{1000}.$$

Therefore $\alpha = \sqrt{1{\cdot}8 \times 10^{-2}} = 0{\cdot}134$

Thus in 1000 litres there is 0·134 g. equivalent H^+ and per litre $1{\cdot}34 \times 10^{-4}$. In dilute solution the activity coefficient may be assumed to be unity, therefore

$$\begin{aligned} \text{pH} = -\log [H^+] &= -\log (1{\cdot}34 \times 10^{-4}) \\ &= -(\bar{4}{\cdot}1271) = 3{\cdot}8729 \\ &= \underline{3{\cdot}87} \end{aligned}$$

Ionic Product of water.—The equilibrium constant for the dissociation of water is given by the equation:

$$K_w = \frac{(H^+)(OH')}{(H_2O)} \qquad . \qquad . \qquad . \qquad (2.8)$$

and since (H_2O) approaches unity in dilute solutions, $K_w \approx (H^+)(OH')$. The constant K_w is called the *ionic product of water*. Conductivity measurements indicate that at 25° the concentration of hydrogen ions is 1×10^{-7} gram equivalent per litre and the concentration of hydroxyl ions is, of course, the same. Hence the ionic product of water at 25° is:

$$K_w = 10^{-7} \times 10^{-7} = 10^{-14}.$$

Consequently the pH value of pure water is 7 at 25° since

$$\text{pH} = -\log (H^+) \approx -\log [H^+] = 7.$$

At 37° $K_w = 2{\cdot}45 \times 10^{-14}$, which gives pH = pOH = 6·80.

There are two different ways in which an acid-base pair may react with water

$$A + H_2O \rightleftharpoons H_3O^+ + B$$
$$B + H_2O \rightleftharpoons \quad A \quad + OH'.$$

The acidic and basic dissociation constants K_a and K_b are, respectively, $K_a = \dfrac{(H_3O^+)(B)}{(A)(H_2O)}$ and $K_b = \dfrac{(A)(OH')}{(B)(H_2O)}$.

$$\text{Eliminating (B)} = \frac{(A)(OH')}{K_b(H_2O)}$$

$$K_a = \frac{(H_3O^+)(OH')(A)}{K_b(H_2O)^2(A)}$$

$$K_a = \frac{(H_3O^+)(OH')}{K_b(H_2O)^2} \approx \frac{K_w}{K_b}$$

$$K_w = K_a K_b \quad . \quad . \quad . \quad (2.9)$$

The basic dissociation constant K_b is now little used and all values are usually reported as K_a. From equation 2.9 it follows that, at 25°

$$pK_w = pK_a + pK_b = 14$$

and

$$pK_a = 14 - pK_b.$$

Thus, K_b can readily be converted to K_a.

THE HENDERSON-HASSELBALCH EQUATION.—Since

$$\text{acid} \rightleftharpoons \text{base} + H^+ \text{ (equation 2.1)}$$

$$\text{i.e. } A \rightleftharpoons B + H^+$$

$$K_a = \frac{(B)(H^+)}{(A)} \text{ and } (H^+) = \frac{K_a(A)}{(B)}.$$

Taking negative logarithms

$$-\log (H^+) = -\log K_a - \log \frac{(A)}{(B)}$$

i.e.

$$pH = pK_a + \log \frac{(B)}{(A)} \quad . \quad . \quad (2.10)$$

This is the *Henderson-Hasselbalch equation.*

Note that this expression employs the activities of B and A, and if concentrations are used then the activity coefficients must be included in the equation

$$pH = pK_a + \log \frac{[B]f_B}{[A]f_A}.$$

Usually $\log f_B/f_A$ is reasonably constant for a given buffer and may be included in the pK_a term when

$$pK_a' = pK_a + \log \frac{f_B}{f_A} \quad . \quad . \quad (2.11)$$

and

$$pH = pK_a' + \log \frac{[B]}{[A]} \quad . \quad . \quad (2.10a)$$

This equation is of importance for the understanding of the action of buffer systems and indicators.

Example 2.4.—The concentration of H_2CO_3 in blood plasma is about 0·00125 M. By use of the Henderson–Hasselbalch equation, calculate the concentration of $BHCO_3$ in the plasma when the pH is 7·4 and also when it is 7·1. H_2CO_3 in blood has a pK_{a1}' value of 6·1.

(Glasgow B.Sc. Single Science Course, 1953.)

$$\text{pH} = pK_{a1}' + \log \frac{[\text{salt}]}{[\text{acid}]}$$

$$= 6{\cdot}1 \quad + \log \frac{[BHCO_3]}{[H_2CO_3]}.$$

Let x be the concentration of $BHCO_3$ in the plasma, then at pH 7·4

$$7{\cdot}4 = 6{\cdot}1 + \log \frac{x}{0{\cdot}00125}$$

$$\log x = 1{\cdot}3 + \log 0{\cdot}00125$$

$$= 1{\cdot}3 + \bar{3}{\cdot}0969$$

$$= \bar{2}{\cdot}3969$$

Therefore $x \approx 0{\cdot}025$ M.

At pH 7·1 $\log x = 1{\cdot}0 + \bar{3}{\cdot}0969$

$$= \bar{2}{\cdot}0969$$

and $x = 0{\cdot}0125$ M.

For polyacids, e.g. H_2CO_3, H_3PO_4, the different acid-base pairs are denoted by $A_1 - B_1$, $A_2 - B_2$, etc., and the dissociation constants are referred to as first, second, third (or primary, secondary, tertiary), K_{a1}, K_{a2}, K_{a3}.

Thus $H_2CO_3 \rightleftharpoons H^+ + HCO_3'$ $\qquad K_{a1} = \dfrac{(H^+)(HCO_3')}{(H_2CO_3)}$

$HCO_3' \rightleftharpoons H^+ + CO_3''$ $\qquad K_{a2} = \dfrac{(H^+)(CO_3'')}{(HCO_3')}$

Hence the general form of the Henderson-Hasselbalch equation may be written as

$$\text{pH} = pK_{a1} + \log \frac{(B_1)}{(A_1)}.$$

Buffer Solutions.—A buffer solution contains a quantity of an acid and its conjugate base such that their concentrations are scarcely altered by proton exchange with other acid-base pairs present. Thus the solution resists any change in pH value. The

pH of the solution is determined by the ratio of base to acid in the buffer pair and its pK_a value.

$$\text{pH} = \text{p}K_a + \log \frac{(\text{B}_{\text{buffer}})}{(\text{A}_{\text{buffer}})}.$$

The choice of a buffer pair should be such that the pH to be stabilized falls within a range equal to p$K_a \pm 1$. For values outside this range a different buffer pair should be selected. This point will be readily appreciated when it is realized that the ratio of base to acid varies from 10 at one pH unit above to 0·1 at one unit below the pK_a value.

All weak acids, in the presence of their conjugate bases, constitute buffer solutions. For example, acetic acid, in the presence of its salt sodium acetate, provides a buffer solution the pH of which is governed by the equation

$$\text{pH} = \text{p}K_a' + \frac{[\text{CH}_3\text{COO}']}{[\text{CH}_3\text{COOH}]}.$$

Since acetic acid is dissociated to a very slight extent whereas sodium acetate is completely ionized, the concentration of acetate ion may be taken equal to the concentration of the salt.

Thus
$$\text{pH} = \text{p}K_a' + \frac{[\text{salt}]}{[\text{acid}]}.$$

The dissociation constant of acetic acid, K_a, is $1{\cdot}8 \times 10^{-5}$; therefore $\text{p}K_a = -\log(1{\cdot}8 \times 10^{-5}) = 4{\cdot}74$, and ideally acetate buffer should be used only to cover the pH range of 3·7 to 5·7. When the salt concentration is equal to that of the acid, as for instance at half neutralization of acetic acid with sodium hydroxide, $\text{pH} = \text{p}K_a'$ and $[\text{H}^+] = K_a'$.

To permit comparison of the efficiency of different buffer solutions Van Slyke (1922) introduced the term *buffer value* which is denoted by β. It is given by the relationship:

$$\beta = \frac{d\text{B}}{d\text{pH}}. \qquad . \qquad . \qquad . \qquad . \qquad (2.12)$$

where dB is the increment (in gram equivalents per litre) of strong base B added to a buffer solution and dpH the resultant increment in pH. An increment of strong acid is equivalent to a negative increment of base, i.e. $-d$B. β always has a positive value since

addition of base increases the pH and addition of acid decreases it, consequently dB and dpH always have the same sign. A solution has a buffer value of unity when one litre will take up 1 gram-equivalent of strong acid or alkali per unit change in pH. Buffer values may be determined directly from a titration curve by reading the values of ΔB and ΔpH for a small increment of B.

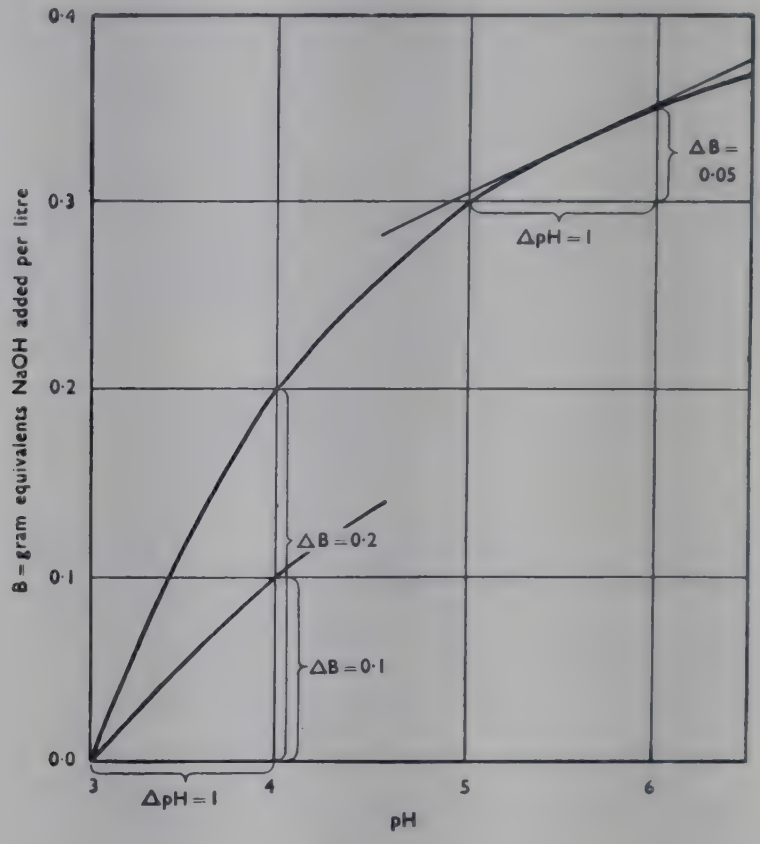

FIG. 2.1

Evaluation of the buffer value β from a titration curve (VAN SLYKE, 1922).

Ideally, the increment should be infinitesimal, but in actual practice measurable increments, provided they are not too large, permit a reasonable evaluation of β. The method is illustrated in Fig. 2.1, and it will be appreciated that values of β may be obtained for any required region of the curve. Alternatively, the tangent to the curve may be drawn at any pH value and its slope evaluated. This, if accurately drawn, gives the exact dB/dpH value and therefore has advantages over the increment method (see Fig. 2.1).

Buffer systems of physiological importance are H_2CO_3: HCO_3', H_2PO_4': HPO_4'' and H-proteinate: B-proteinate (where B = cation).

INDICATORS.—An indicator is an acid-base pair where the acid and base forms have different colours. A very small quantity of the indicator is added to the solution under investigation so that the concentrations of other acid-base pairs are largely unaffected. The resultant colour of the solution is measured by comparator or colorimeter and the ratio of the concentrations of base and acid forms of the indicator thereby determined. Knowing the pK_a value of the indicator, the pH value of the solution may be calculated from equation 2.10. Indicators, as with buffer solutions, should be used only within the range $pK_a \pm 1$.

Some indicators have only one coloured form, e.g. the acid form of phenolphthalein is colourless and the base form red, whereas others have both forms coloured, e.g. methyl red, which has acid and base forms red and yellow respectively.

The Dipolar Ion Form of Amino Acids.

An amino acid ionizes according to the pH of its solution and the following stages may be recognized:

$$RCH\overset{+}{N}H_3COOH \rightleftharpoons RCH\overset{+}{N}H_3COO' \rightleftharpoons RCHNH_2COO'$$

	acidic	isoelectric	alkaline
Net charge	+1	0	−1

The isoelectric species, which bears no net charge, is a dipolar ion. Formerly it was believed that the isoelectric amino acid had the structure $RCHNH_2COOH$, but evidence from various physical and chemical sources, such as spectroscopy, dielectric constant and titration in organic solvents, indicated that the dipolar ion is the correct representation. Amino acids in the crystalline state have high melting points which indicate that they also exist as the doubly charged molecule in the solid state.

The dipolar ion possesses two ionizing groups and is therefore characterized by two pK_a values. Consider the simplest amino acid, glycine, which is aminoacetic acid. In Fig. 2.2 the titration curves of acetic acid and glycine are compared. When acetic acid is titrated with sodium hydroxide there is a point of inflexion at half neutralization which corresponds to a pH value of 4·74. This, as already mentioned, is the pK_a value for acetic acid since at half neutralization $pH = pK_a$. It will be noticed that the pH of the solution changes very little during the addition of 0·2 to

0·8 equivalents of alkali; herein lies the basis of the buffering power of such solutions, to which reference has already been made. The titration curve of glycine with acid and alkali displays two points of inflexion, at pH 2·4 and 9·7. These are referred to as pK_{a1} and pK_{a2} respectively, the former being due to the carboxyl group and the latter to the amino group. Note the convention that pK_a values are numbered from the acid to the alkaline region.

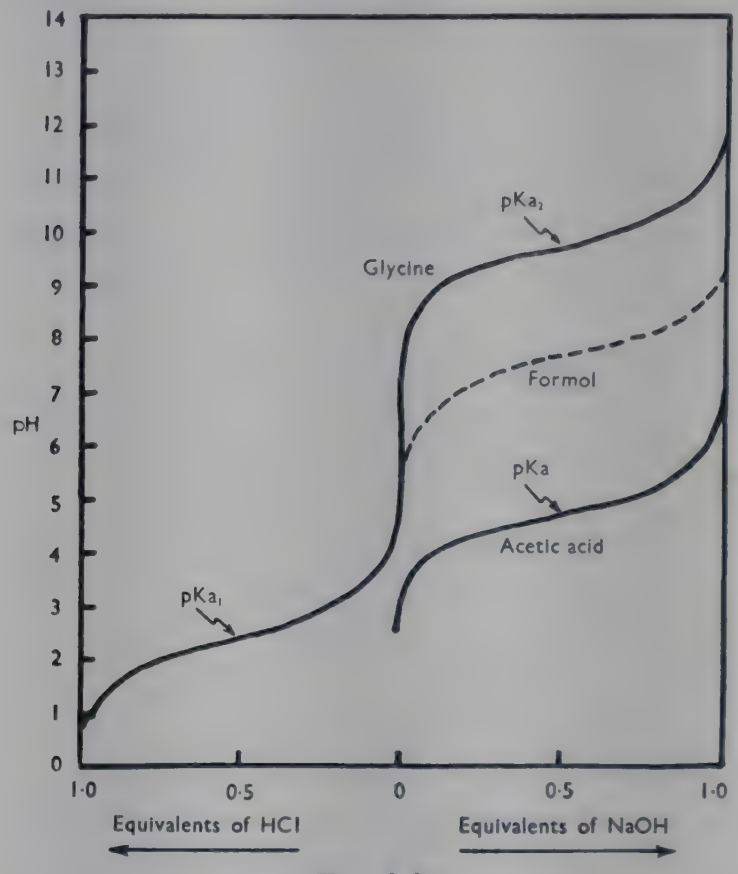

FIG. 2.2

Titration curves of glycine and acetic acid. Note the effect of addition of formaldehyde in the formol titration.

The fact that pK_{a2} for glycine is due to the amino group may be demonstrated by a procedure, developed by Sörensen, known as the *formol* titration. If formaldehyde is added to an amino acid, either one or two molecules react with the amino group to form methylol derivatives. The equilibria set up may be represented as follows:

$$`OOCRCH\overset{+}{N}H_3 \rightleftharpoons `OOCRCHNH_2 + H^+$$

$$`OOCRCHNH_2 + HCHO \rightleftharpoons `OOCRCHNHCH_2OH$$

$$`OOCRCHNH_2 + 2HCHO \rightleftharpoons `OOCRCHN(CH_2OH)_2$$

Titration of the resultant solution with alkali reveals that the titration curve has shifted appreciably towards the acid side and the stoichiometric end-point now occurs at approximately pH 9 instead of 12. As pH 9 falls within the range of phenolphthalein, this indicator may be used for the titration. Hence pK_{a_2} at 9·7 for glycine must be due to the amino group. The formol titration is a useful method for determining the amino groups of aliphatic amino acids and polypeptides, and for a discussion of the principle of this method the reader is referred to Clark (1952).

All monoaminomonocarboxylic acids which possess no other ionizable group have two pK_a values which do not differ greatly from those of glycine, but with monoaminodicarboxylic and diaminomonocarboxylic acids there are three pK_a values. For instance, aspartic acid has pK_a values for the two carboxyl groups, the α (pK_{a_1}) being slightly more acidic than the β group (pK_{a_2}). The values are 1·9 and 3·7 respectively, and on account of their close proximity the points of inflexion on the titration curve are not well resolved. pK_{a_3} for the amino group of aspartic acid occurs at 9·6. Lysine has $pK_{a_1} = 2{\cdot}2$, $pK_{a_2} = 8{\cdot}9$ and $pK_{a_3} = 10{\cdot}5$ for the carboxyl, α- and ϵ-amino groups respectively and the ϵ-amino group is therefore a slightly stronger base than the α group.

Some amino acids possess titratable groups other than the carboxyl and amino groups. The phenolic hydroxyl of tyrosine, sulphydryl of cysteine, guanidino of arginine and the imidazolyl group of histidine behave in this way, the former two as acids and the latter two as bases. Histidine, for example, has $pK_{a_1} = 1{\cdot}8$ for the carboxyl, $pK_{a_2} = 6{\cdot}1$ for the imidazole and $pK_{a_3} = 9{\cdot}2$ for the amino group.

Isoelectric and Isoionic Points.—The isoelectric point is the pH at which the ionic species bears no net charge and consequently does not migrate in an electric field. The majority of proteins display their minimum solubility at the isoelectric point. The isoionic point is defined as the pH at which the number of protons dissociated from acidic groups is equal to the number of protons combined with basic groups. Note particularly that the isoionic point refers to the dissociation and combination of protons only and that the isoelectric and isoionic points are therefore identical only if the dipolar ion combines solely with protons. The isoelectric point is usually denoted by the symbol pI

and for an amino acid having two pK_a values is obtained as follows

$$pI = \frac{pK_{a1} + pK_{a2}}{2} \quad . \quad . \quad (2.13)$$

For amino acids bearing three dissociating groups it is customary to take into account only the two predominating pK_a values, i.e. pK_{a1} and pK_{a2} for monoaminodicarboxylic acids and pK_{a2} and pK_{a3} for diaminomonocarboxylic acids. The following example illustrates this point.

Example 2.5.—Calculate the isoelectric point of (*a*) glycine, (*b*) arginine. Glycine has $pK_{a_1} = 2{\cdot}4$ and $pK_{a_2} = 9{\cdot}7$ and arginine has $pK_{a_1} = 2{\cdot}0$, $pK_{a_2} = 9{\cdot}0$ and $pK_{a_3} = 12{\cdot}5$.

(*a*) *Glycine.* $pI = \frac{2{\cdot}4 + 9{\cdot}7}{2} = \underline{6{\cdot}05}$.

(*b*) *Arginine.* The three pK_a values refer to the carboxyl, α-amino and guanidino groups respectively. The latter two basic groups predominate and the isoelectric point is given by

$$pI = \frac{9{\cdot}0 + 12{\cdot}5}{2} = \underline{10{\cdot}75}.$$

Electrolyte Behaviour of Proteins.

Proteins consist of amino acid residues condensed together and their titration behaviour is of considerable interest. Since α-amino and carboxyl groups are used for the formation of peptide bonds, it follows that the polar groups of the amino acid side-chains must provide the major contribution to electrolyte behaviour. The β- and γ-carboxyls of aspartic and glutamic acids respectively, ε-amino of lysine, phenolic hydroxyl of tyrosine, guanidino group of arginine, imidazolyl group of histidine and sulphydryl group of cysteine all contribute in this way. Consequently the actual shape of the titration curve of a protein will depend on the number and type of polar side-chain groups it possesses and on their pK_a values.

From titration data it is possible to make an approximate calculation of the number of side-chain groups of each type present in the protein molecule if the basic assumption is made that the pK_a values of the groups in the side-chain do not differ appreciably from the values characteristic of the free amino acids. Considerable success has been achieved in this way, as confirmed by subsequent hydrolysis and analysis of the protein.

Titration curves reveal that proteins possess great buffering capacity; relatively large quantities of acid and alkali may be added without producing appreciable change in pH. This property is of enormous importance in biological systems where it is found that proteins constitute the main buffering system. For example, about 80 per cent. of the total buffering power of mammalian blood is due to proteins.

The Donnan Membrane Equilibrium.

The net electric charge of proteins gives rise to their effect in producing an unequal distribution of diffusible ions on either side of a membrane through which the proteins cannot pass.

Donnan, in 1911, propounded the theory of membrane equilibria which accounts for the effect of electrical charges on macromolecules. Donnan equilibria, as they are called, occur whenever a charged molecule is constrained in its movements, as, for example, when a membrane is impermeable to it. The behaviour of charged protein molecules is of great interest in biochemistry. Consider, therefore, the sodium salt of a protein at concentration c_1 separated from a solution of sodium chloride of concentration c_2 by a membrane impermeable to protein molecules but permeable to inorganic ions. For convenience assume the two solutions to be of equal volume. There will be a concentration c_1 of protein molecules bearing a net negative charge Pr′ and an equivalent concentration c_1 of Na^+ ions. This is illustrated diagrammatically in Fig. 2.3(*a*) where the protein is in compartment A. In compartment B is sodium chloride solution at concentration c_2 and hence there is a concentration gradient of Cl′ ions in response to which some diffuse across the membrane into compartment A.

	A		B	
(*a*) Initial state	Na^+	c_1	Na^+	c_2
	Pr′	c_1	Cl′	c_2
(*b*) Equilibrium state	Na^+	$c_1 + x$	Na^+	$c_2 - x$
	Pr′	c_1	Cl′	$c_2 - x$
	Cl′	x		

FIG. 2.3

Initial and equilibrium states in a Donnan equilibrium. Equivalent concentration x of NaCl has been transferred from compartment B to compartment A.

As this would upset the electrical neutrality of the system they are accompanied by an equivalent concentration of Na^+ ions. This, in its turn, sets up a concentration gradient of Na^+ ions which begin to diffuse back from compartment A into B. The two processes continue until equilibrium is set up when the concentration gradient of Cl′ from B to A is balanced by that of the Na^+ from A to B. At equilibrium consider that a concentration x of Cl′ has diffused into compartment A. The equilibrium state will then be that depicted in Fig. 2.3(*b*).

At constant temperature and pressure the free energy necessary to transfer one mole of Cl′ from compartment B to A in a reversible manner is

$$\Delta G = RT \ln \frac{(\mathrm{Cl}')_\mathrm{A}}{(\mathrm{Cl}')_\mathrm{B}},$$

and similarly for one mole of Na^+

$$\Delta G = RT \ln \frac{(\mathrm{Na}^+)_\mathrm{A}}{(\mathrm{Na}^+)_\mathrm{B}}.$$

From thermodynamics we know that the total free energy change is zero at equilibrium (Chapter III).

Therefore $$RT \ln \frac{(\mathrm{Cl}')_\mathrm{A}}{(\mathrm{Cl}')_\mathrm{B}} + RT \ln \frac{(\mathrm{Na}^+)_\mathrm{A}}{(\mathrm{Na}^+)_\mathrm{B}} = 0$$

and $$\frac{(\mathrm{Cl}')_\mathrm{A}}{(\mathrm{Cl}')_\mathrm{B}} = \frac{(\mathrm{Na}^+)_\mathrm{B}}{(\mathrm{Na}^+)_\mathrm{A}} \quad . \quad . \quad . \quad (2.14)$$

This is a general expression and holds for all univalent cations and anions.

Therefore $$\frac{x}{c_2 - x} = \frac{c_2 - x}{c_1 + x}$$

and $$x = \frac{{c_2}^2}{c_1 + 2c_2}$$

Thus the ratio of NaCl in compartment B to that in compartment A is

$$\frac{(\mathrm{NaCl})_\mathrm{B}}{(\mathrm{NaCl})_\mathrm{A}} = \frac{(\mathrm{Cl}')_\mathrm{B}}{(\mathrm{Cl}')_\mathrm{A}} = \frac{c_2 - x}{x} = 1 + \frac{c_1}{c_2} \quad . \quad . \quad . \quad (2.15)$$

which means that the greater the equivalent concentration of

protein c_1, the more uneven will be the final distribution of diffusible ions. However, if the salt concentration c_2 is high, its distribution at equilibrium will approach unity and the Donnan effect will be negligible. Practical use is made of this fact in osmotic pressure measurements (p. 9). Establishment of a membrane equilibrium results in osmotic pressures that are too high being measured and hence introduces error. It can be largely eliminated by working at the isoelectric point when the protein bears no net charge or, alternatively, by employing a concentrated salt solution as solvent for the protein.

For polyvalent cations and anions the general expression for the Donnan distribution becomes

$$\left(\frac{(\text{Anion})_A}{(\text{Anion})_B}\right)^{\frac{1}{z_a}} = \left(\frac{(\text{Cation})_B}{(\text{Cation})_A}\right)^{\frac{1}{z_c}}$$

where z_a and z_c are the valencies of the anions and cations respectively.

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PROBLEMS

2.1. Calculate the hydrogen ion concentration of solutions of pH value 6·0 and 9·5.

2.2. What is the pH value of 0·5 per cent. (w/v) hydrochloric acid? (Assume complete dissociation.)

2.3. Calculate the pH value of (*a*) M/1000 sulphuric acid and (*b*) M/1000 sodium hydroxide, assuming both solutions to be completely ionized. What would be the resultant pH value of the solution if 25 ml. of the above sulphuric acid were added to 20 ml. of the sodium hydroxide solution?

2.4. 10 ml. of a M/20 solution of potassium dihydrogen phosphate are added to 10 ml. of a solution of disodium hydrogen phosphate. The pH of the resulting solution is 6·725. Calculate the molarity of the disodium hydrogen phosphate if the second dissociation constant for phosphoric acid is $1{\cdot}4 \times 10^{-7}$.

2.5. Calculate the ionic strength of the following solutions: (*a*) 0·1 N NaOH; (*b*) 2 N H_2SO_4, (*c*) 0·025 M $MgSO_4$; (*d*) 0·02 M KH_2PO_4; (*e*) 0·15 M $CaCl_2$; (*f*) 0·5 M Na_2HPO_4; (*g*) 0·075 M CH_3COONa. Complete dissociation may be assumed in each case. Derive general expressions for the relation between ionic strength and molar concentration for (1) uni-univalent, (2) uni-divalent, (3) di-divalent, (4) uni-trivalent and (5) di-trivalent salts, and use the derivations to check the above calculations.

2.6. Calculate the ionic strength of 0·01 N acetic acid if the dissociation constant of the acid is $1{\cdot}8 \times 10^{-5}$.

2.7. Calculate the activity coefficients and activities of the ions in aqueous solutions of (*a*) 5 mM H_2SO_4 (*b*) 2 mM NaCl.

2.8. A solution is 5 mM with respect to $MgCl_2$ and 2·5 mM with respect to $MgSO_4$. Determine the ionic strength and estimate the activities of the ions.

2.9. The primary dissociation constant for carbonic acid is $4{\cdot}31 \times 10^{-7}$. If the pH of a sample of blood is 7·45, determine the ratio of carbonic acid to bicarbonate present.

2.10. Determine the secondary dissociation constant of phosphoric acid if blood of pH 7·4 contains 12·85 mg. HPO_4'' and 3·21 mg. H_2PO_4' per 100 ml. plasma respectively.

2.11. The acid dissociation constants of haemoglobin and oxyhaemoglobin are respectively $6{\cdot}6 \times 10^{-9}$ and $2{\cdot}4 \times 10^{-7}$. If the normal blood pH value is accepted as 7·4 and both these compounds are present as free acids and as salts, determine which is the more efficient buffering system in the blood.

What is the ratio of free acid to salt for oxyhaemoglobin and haemoglobin in such blood?

2.12. Determine the isoelectric points of the following amino acids at 25°. The values for the pK_a values given refer to this temperature.

(*a*) α-Alanine: $pK_{a_1} = 2{\cdot}34$, $pK_{a_2} = 9{\cdot}69$.

(*b*) β-Alanine: $pK_{a_1} = 3{\cdot}60$, $pK_{a_2} = 10{\cdot}19$.

(*c*) Asparagine: $pK_{a_1} = 2{\cdot}02$, $pK_{a_2} = 8{\cdot}80$.

(*d*) Cystine: $pK_{a_1} = 1{\cdot}65$, $pK_{a_2} = 2{\cdot}26$, $pK_{a_3} = 7{\cdot}85$, $pK_{a_4} = 9{\cdot}85$.

(*e*) Isoleucine: $pK_{a_1} = 2{\cdot}36$, $pK_{a_2} = 9{\cdot}68$.

(*f*) Sarcosine: $pK_{a_1} = 2{\cdot}23$, $pK_{a_2} = 10{\cdot}01$ ($CH_2\overset{+}{N}H_2$).

(*g*) L-Tyrosine: $pK_{a_1} = 2{\cdot}20$, $pK_{a_2} = 9{\cdot}11$, $pK_{a_3} = 10{\cdot}07$ (OH).

(*h*) Taurine: $pK_{a_1} = 1{\cdot}5$ (SO_3H), $pK_{a_2} = 8{\cdot}74$.

2.13. Determine the isoelectric points of the following dipeptides at 25°. The pK_a values given refer to this temperature.

(*a*) Glycylglycine: $pK_{a_1} = 3{\cdot}06$, $pK_{a_2} = 8{\cdot}13$.

(*b*) Tyrosyltyrosine: $pK_{a_1} = 3{\cdot}52$, $pK_{a_2}' = 7{\cdot}69$, $pK_{a_3} = 9{\cdot}80$, $pK_{a_4} = 10{\cdot}26$. pK_{a_3} and pK_{a_4} refer to the hydroxyl groups.

(*c*) Aspartylaspartic acid: $pK_{a_1} = 2{\cdot}70$, $pK_{a_2} = 3{\cdot}40$, $pK_{a_3} = 4{\cdot}70$, $pK_{a_4} = 8{\cdot}26$. pK_{a_1} and pK_{a_3} refer to the β-carboxyl groups.

(*d*) Histidylhistidine: $pK_{a_1} = 2{\cdot}25$, $pK_{a_2} = 5{\cdot}60$, $pK_{a_3} = 6{\cdot}80$, $pK_{a_4} = 7{\cdot}80$. pK_{a_2} and pK_{a_3} refer to the imidazolyl groups.

(*e*) Glycylalanine: $pK_{a_1} = 3{\cdot}15$, $pK_{a_2} = 8{\cdot}25$.

(*f*) Glycyltyrosine: $pK_{a_1} = 2{\cdot}98$, $pK_{a_2} = 8{\cdot}40$, $pK_{a_3} = 10{\cdot}40$ (OH group).

(*g*) Aspartylglycine; $pK_{a_1} = 2{\cdot}10$, $pK_{a_2} = 4{\cdot}53$ (β-carboxyl), $pK_{a_3} = 9{\cdot}07$.

2.14. Hastings and Van Slyke obtained the following results for the electrometric titration of 0·1 M citric acid with sodium hydroxide:

NaOH (moles/litre)	0·0000	0·0197	0·0395	0·0592	0·0790	0·0987	0·1183
pH	2·06	2·51	2·88	3·14	3·42	3·67	3·98
NaOH (moles/litre)	0·1381	0·1578	0·1775	0·1973	0·2170	0·2368	0·2564
pH	4·20	4·46	4·69	4·94	5·18	5·42	5·70
NaOH (moles/litre)	0·2762	0·2782	0·2802	0·2821	0·2861	0·2900	0·2939
pH	6·06	6·07	6·14	6·17	6·32	6·54	6·83

Plot the titration curve and obtain the buffer value for the region of maximum buffering power. Over what range may the solution be used as an efficient buffer system?

(Data from HASTINGS & VAN SLYKE (1922), *J. biol. Chem.*, **53**, 269.)

2.15. The following data were obtained in the electrometric titration of 1 mole of potassium dihydrogen phosphate with concentrated sodium hydroxide. The KH_2PO_4 was contained in 1 litre of solution and the volume change due to the addition of the NaOH may be ignored.

Moles NaOH added per mole KH_2PO_4	0·000	0·040	0·125	0·200	0·300	0·475	0·590
Determined pH	4·00	5·50	6·00	6·25	6·45	6·75	7·00
Moles NaOH added per mole KH_2PO_4	0·700	0·800	0·850	0·900	0·940	1·000	
Determined pH	7·20	7·40	7·65	7·80	8·00	9·90	

Determine the second dissociation constant of phosphoric acid and assess the buffer value at the pH corresponding to pK_{a_2}. How does this buffer value compare with those for the KH_2PO_4:$NaKHPO_4$ system at pH 6·0 and 7·5?

2.16. A plasma sample gave the following analysis: total CO_2 concentration 27 m-moles/litre and bicarbonate concentration 25·7 m-moles/litre. If pK_{a_1}' for carbonic acid is 6·11, calculate the pH value of the plasma.

2.17. The pH of plasma samples of arterial and venous blood were 7·45 and 7·38. Analysis for the total CO_2 content of each gave 59·1 and 62·1 volume per cent. respectively. Calculate the partial pressure of CO_2, its concentration in the plasma and the plasma bicarbonate concentration in millimoles per litre for the samples of arterial and venous blood. $pK_{a_1}' = 6{\cdot}11$ for carbonic acid and the solubility factor for CO_2, $K_0 = 0{\cdot}0301$ when the concentrations are expressed as millimolar and p_{CO_2} in mm. Hg. *Note:* [Dissolved CO_2 + H_2CO_3] = $K_0 p_{CO_2}$.

(Consultation of p. 153 may assist in solving this problem.)

2.18. Human red blood cells at pH 7·17 and 37° were in contact with a partial pressure of 25·4 mm. CO_2 when oxygenated, and 48·4 mm. CO_2 after the oxygen was given up at the same pH. If pK_a' for oxygenated and non-oxygenated red blood cells is 6·070 and 6·018 respectively, calculate the total CO_2 concentration present in the cells. The solubility factor for CO_2 in the cells at 37° is 0·0362 when concentrations are expressed as millimolar and p_{CO_2} in mm. Hg.

(Data from DILL, DALY & FORBES (1937), *J. biol. Chem.*, **117**, 569.)

2.19. The following data were obtained in experiments on samples of oxygenated and non-oxygenated red blood cells of the ox.

Oxygenated: $p_{CO_2} = 37{\cdot}3$ mm.; pH = 7·22; $pK_a' = 6{\cdot}053$

Non-oxygenated: $p_{CO_2} = 50{\cdot}7$ mm.; pH = 7·22; $pK_a' = 6{\cdot}022$

These figures refer to 37° at which temperature the solubility factor (K_0) for CO_2 in red blood cells is 0·0362 when concentrations are expressed as millimolar and p_{CO_2} in mm. Hg. Calculate the bicarbonate concentration in each sample.

(Data from DILL, DALY & FORBES (1937), *J. biol. Chem.*, **117**, 569.)

2.20. The following titration data refer to horse haemoglobin (Hb) and oxyhaemoglobin (HbO_2) in the presence of 0·333 M NaCl:

Haemoglobin		*Oxyhaemoglobin*	
Acid (−) *or base* (+) *per gram Hb* m-equivalent	*pH*	*Acid* (−) *or base* (+) *per gram* HbO_2 m-equivalent	*pH*
−0·514	4·280	−0·514	4·280
−0·452	4·415	−0·453	4·410
−0·419	4·525	−0·420	4·525
−0·390	4·610	−0·392	4·618
−0·323	4·842	−0·324	4·860
−0·258	5·160	−0·259	5·188
−0·224	5·320	−0·225	5·430
−0·172	5·690	−0·173	5·800
−0·130	6·072	−0·130	6·055
−0·064	6·541	−0·063	6·430
0·0	6·910	+0·001	6·795
+0·070	7·295	+0·072	7·130
+0·131	7·660	+0·133	7·510
+0·171	7·860	+0·172	7·725
+0·208	8·140	+0·209	8·043
+0·254	8·545	+0·254	8·450
+0·288	8·910	+0·288	8·890
+0·311	9·130	+0·292	8·990
+0·331	9·350	+0·311	9·130
+0·350	9·410	+0·331	9·355
+0·357	9·465	+0·350	9·410
+0·407	9·800	+0·357	9·480
		+0·407	9·800

Plot the titration curves for haemoglobin and oxyhaemoglobin and from these deduce which form of haemoglobin is the stronger acid.

(After GERMAN & WYMAN (1937), *J. biol. Chem.*, **117**, 533.)

2.21. In a series of experiments the sodium salt of a protein at equivalent concentration 0·1 was separated from an equal volume of sodium chloride solution by a semi-permeable membrane. If the equivalent concentrations of salt solution were 0·005, 0·010, 0·055, 0·120 and 1·20, calculate the ratios of the distribution of electrolyte at equilibrium in each case.

2.22. The effect of ionic strength on the first dissociation constant of carbonic acid has been investigated and the following data obtained.

Solutions were prepared from purified $NaHCO_3$ and NaCl and were equilibrated at 38° with hydrogen and CO_2 at a tension previously calculated to give the desired pH. Samples of the gas phase were analysed for total CO_2 content

in the Van Slyke manometric gas apparatus. The pH of the solution was measured electrometrically at 38°.

Experiment Number	p_{CO_2} *mm.* Hg	*Solubility coefficient of* CO_2 (α)	*Total* CO_2 *concentration* mM	*pH*	*NaCl concentration* mM
1	61·6	0·552	12·25	6·977	0·00
7	158·1	0·546	35·10	6·976	24·83
9	152·3	0·544	35·12	6·970	49·65
11	145·0	0·541	34·75	6·970	74·43
13	140·5	0·538	34·86	6·970	99·34
15	136·8	0·532	34·60	6·950	149·00

From this information calculate the concentration of carbonic acid and thence the pK_{a_1}' value for each experiment. Plot the values of pK_{a_1}' so obtained against the square root of the ionic strength (I) and from the resulting graph deduce the relationship between K_{a_1}' and $\sqrt{I}$ and hence the relationship between I and the activity coefficient of the bicarbonate ion.

Note: Where concentrations are expressed as millimolar and p_{CO_2} as mm. Hg:

$$pK_{a_1}' = pH - \log [NaHCO_3] + \log p_{CO_2} + \log \frac{\alpha}{760 \times 0{\cdot}0224}$$

assuming $NaHCO_3$ to be completely dissociated and hence $[NaHCO_3] = [HCO_3']$.

(After HASTINGS & SENDROY (1925), *J. biol. Chem.*, **65**, 445.)

2.23. A solution of the sodium salt of a non-diffusible substance is separated from an equal volume of a solution of sodium chloride by a semi-permeable membrane. For convenience the compartments will be referred to as A and B respectively. Calculate the concentrations of sodium and chloride ions in compartments A and B at equilibrium when the initial concentrations are as follows:

Initial concentrations (mM)

Non-diffusible substance (compartment A)	*Sodium chloride (compartment B)*
0·01	1·00
1·00	1·00
1·00	0·50
1·00	0·01

(After CLARK (1952), *Topics in Physical Chemistry*, 2nd ed., p. 153. Baltimore: Williams & Wilkins Co.)

2.24. 40 ml. of a 0·000650 M solution of sulphadiazine were titrated with 0·0500 N NaOH from a micro-burette; the pH value was measured with a glass electrode, some of the results being as follows:

Titre (ml.)	0·139	0·279	0·350
pH value	6·17	6·64	6·90

Calculate the pK_a value for sulphadiazine, neglecting the slight change in total volume during the titration.

(After SPEAKMAN, unpublished data.)

CHAPTER III

THERMODYNAMICS

Broadly, the study of chemical reactions may be approached from an analysis of the behaviour of the participating molecules, as in Chemical Kinetics, or from a consideration of the energy transfers that accompany the reactions, as in Chemical Thermodynamics. The latter science deals with the initial and final states of systems and, in general, is not concerned with the speed at which changes occur. Although thermodynamics has contributed to our understanding of processes at the molecular level, the science has developed from laws deduced directly from experience of the behaviour of matter in bulk, and not from the molecular theory of matter.

The First Law of Thermodynamics is concerned with the principle of conservation of energy. It is only possible to convert one form of energy into another form; energy can be neither created nor destroyed.[1] This can be expressed in symbols as follows:

If W is the mechanical work done by the system, Q the quantity of heat absorbed by the system, and ΔU its change in internal energy, then

$$\Delta U = Q - W \quad . \quad . \quad . \quad (3.1)$$

The quantity U, which is called the *internal* or *intrinsic* energy, includes all forms of energy of the system except those resulting from its position in space. The absolute value of U cannot be determined, but we are concerned only with changes in U such as occur when a chemical reaction takes place. In changing from state 1 to state 2 the change in internal energy is given by

$$\Delta U = U_2 - U_1 \quad . \quad . \quad . \quad (3.2)$$

In this equation use is made of a notation which finds general application in this subject: namely ΔU to denote an *increase* in

[1] Energy changes in biochemical reactions are never, of course, of such magnitude as make the principle of equivalence of mass and energy (Einstein) relevant to these systems.

U of the system, ΔV for an increase in V, and so on. If the change had given rise to a decrease in the value considered, the delta sign would be preceded by a minus. It is important to note that ΔU is independent of the pathway by which the change is carried out; it depends only upon the initial and final states, and U is known, therefore, as an intrinsic thermodynamic variable. If this were not so it would be possible to create energy from the system and thus violate the First Law. Hess's Law, used in thermochemical calculations, is a restatement of this law as it applies to heats of reaction, although historically Hess's Law preceded it. Q and W, however, are dependent on the pathway of the change.

A special case occurs when no external work is done (i.e. $W = 0$). This is usually covered by saying that the process occurs at *constant volume*, although this does not exclude the possibility that other forms of external work *might* occur. Then:

$$Q_v = \Delta U \quad . \quad . \quad . \quad (3.3)$$

where the subscript $_v$ indicates constant volume. Thus, at constant volume, ΔU is equal to the observed heat change and, by the convention mentioned, is negative for an exothermic reaction (i.e. energy, as heat, lost to the system) and positive for an endothermic reaction (heat gained by the system).

A reaction involving an increase of volume and occurring at *constant pressure* (as in the case of reactions in open vessels) necessarily implies the performance of work against the surrounding atmosphere. Biochemical reactions usually occur at a constant pressure, that of the atmosphere, and not at constant volume. If the volume increases, a proportion of the intrinsic energy change will be used to perform work against the pressure of the atmosphere and, in consequence, all of the energy liberated will not appear as heat of reaction. It is convenient to make use of another quantity, H, the *heat content* or *enthalpy*, which is related to U by the equation

$$H = U + PV \quad . \quad . \quad . \quad (3.4)$$

Here P is constant and V, the volume of the system, has, like U, a defined value for any given state; H, therefore, is also an intrinsic thermodynamic variable. The relation between changes

in U and H is readily established. If V_1 is the initial and V_2 the final volume

$$W = PV_2 - PV_1 = P\Delta V.$$

Therefore $$\Delta U = Q_p - P\Delta V \quad . \quad . \quad . \quad . \quad (3.5)$$

and $$Q_p = \Delta U + P\Delta V = \Delta H \quad . \quad . \quad (3.6)$$

At constant pressure then, ΔH is the heat absorbed in the reaction. Values for ΔH may be obtained calorimetrically, but since the bomb calorimeter is a constant volume device, the heats of reaction so measured give changes in internal energy. Values of ΔU, however, may readily be converted to ΔH. If a reaction between gases is represented by

$$A \rightarrow B + C + D,$$

there is an increase of two in the total number of molecules in the system when it occurs at any temperature T. Since $PV = nRT$, where n is the number of moles, the work done due to the increase in volume $P\Delta V$ is therefore $2RT$ and $\Delta H = \Delta U + 2RT$. Volume changes when extra molecules of solids or liquids arise are negligible compared with those for gases. Hence, in the example given, if A were a solid, B a liquid and C and D both gases, the work done due to the increase in volume would also be $2RT$. Some examples of complete combustion are:

1. Solid glucose to liquid water and gaseous carbon dioxide. (Note that the subscripts S, L and G refer to the state.)

$$C_6H_{12}O_{6(S)} + 6O_{2(G)} \rightarrow 6CO_{2(G)} + 6H_2O_{(L)}$$

$\Delta H = -673{,}000$ calories or -673 kilocalories, i.e. an exothermic reaction.

2. Solid maltose to liquid water and gaseous carbon dioxide.

$$C_{12}H_{22}O_{11(S)} + 12O_{2(G)} \rightarrow 12CO_{2(G)} + 11H_2O_{(L)}$$

$\Delta H = -1{,}350$ kilocalories.

3. Solid urea to liquid water and gaseous carbon dioxide and nitrogen.

$$CO(NH_2)_{2(S)} + 1{\cdot}5O_{2(G)} \rightarrow CO_{2(G)} + N_{2(G)} + 2H_2O_{(L)}$$

$\Delta H = -152$ kilocalories.

Example 3.1.—The combustion of solid urea to liquid water and gaseous carbon dioxide and nitrogen in a bomb calorimeter at 25° liberates 152·3 kilocalories. Determine the heat content change for the reaction.

As will be seen from case 3 above, the increase in volume attendant on this reaction is $2 - 1{\cdot}5 = 0{\cdot}5$ mole of gas.

Hence
$$\begin{aligned}\Delta H &= \Delta U + 0{\cdot}5RT \\ &= -152300 + 0{\cdot}5 \times 1{\cdot}987 \times 298 \\ &= -152300 + 296 = -152{,}004 \text{ calories} \\ &= \underline{-152{\cdot}0 \text{ kilocalories.}}\end{aligned}$$

The Second Law.—Kelvin stated this law as follows: 'It is impossible to take heat from a system and convert it into work without other simultaneous changes occurring in the system or in its environment.' Lewis has given an alternative statement of the law in the words: 'Every process that occurs spontaneously is capable of doing work; to reverse any such process requires the expenditure of work from outside.'

Although in particular instances, such as the oxidation of hydrocarbons in the internal-combustion engine, the ability of a chemical reaction to perform work can readily be appreciated, for most of the reactions encountered in the laboratory difficulties are presented. Reference should be made to a textbook of chemical thermodynamics (that of Butler (1951) is particularly recommended on this point) as to how the conversion of energy of reaction into work can be visualized and evaluated. Meanwhile, the contribution of the Second Law towards the problem of assessing chemical affinity—the 'driving force' of reactions—may be noted. A great impetus was given to thermochemical research in the last century by the belief that the heat of a reaction measured its affinity: the greater the amount of energy liberated, the more affinity the reactants possessed for each other. This view was rendered untenable by the discovery of endothermic reactions which could proceed, and so exhibit 'affinity', although they absorbed heat in the process. When, however, attention was focused on the work that a reaction was able to perform rather than on the heat that it liberated, such difficulties did not arise, for the Second Law states that if a reaction is inherently incapable of work it will not be spontaneous; that is, will not proceed of its own accord and will exhibit no chemical affinity. Negative values for the work done by a spontaneous reaction, in contrast to negative values for heat liberated, will not be encountered. The

extent to which a reaction can proceed before it reaches equilibrium is hence related to the energy which is capable of conversion into work when the reaction takes place reversibly, i.e. the maximum work. There is another intrinsic thermodynamic variable known as the *free energy* (and defined, as seen later, by equation 3.7, namely $G = H - TS$, where G is the symbol denoting free energy), the change in which, during a reaction occurring at constant temperature and pressure, is equal to this maximum work.

Another concept arising from the Second Law is that of *entropy*, denoted by S. If at a fixed temperature T an amount of heat Q is absorbed reversibly by a system, its entropy rises by Q/T. Entropy is an intrinsic thermodynamic function most readily appreciated by considering the changes that occur when entropy alters. Thus, if the transfer of heat were reversible, that is, if the heat flowed from a system that was at a higher temperature than its surroundings only by an infinitesimally small amount, then the loss of entropy by the system would equal the gain by the surroundings. But, in actual fact, the system will always be at a definitely higher temperature; perfectly reversible systems are not encountered in Nature, and Q/T for the system will be less than for the surroundings. For system and surroundings taken together there will have been an overall increase in S. Now, heat flows from a higher to a lower temperature spontaneously, and as a consequence of the levelling out of the temperature difference, there is a destruction of an ordered arrangement. Thus, if the transfer of heat occurred between two gases, there would be originally a segregation of fast-moving molecules, at the higher temperature, from molecules that move more slowly on the average. This distinction is abolished when the temperatures are equated, and as a result the energy becomes distributed in a more random manner. Fast-moving molecules of high energy are no longer segregated from those with lower energies. Spontaneous changes occur, in general, with an increase in disorder, and this is on a parallel with an increase in S. Another view which may assist the visualizing of entropy is to regard the intrinsic energy as made up of the energy of chemical linkage that is liberated as free energy in reactions, plus the random energy of vibration and rotation of atoms and molecules. Certain molecules, in particular those that are complicated, have a high capacity for taking up this type of

random disordered energy and they have a high entropy. The relation between free energy, heat content and entropy is

$$G = H - TS \quad . \quad . \quad . \quad (3.7)$$

Thus, if a system is changed from one state to another at the same temperature, the change in free energy associated with this change is

$$\Delta G = \Delta H - T\Delta S \quad . \quad . \quad . \quad (3.8)$$

Since G is an intrinsic property, ΔG is independent of the path of the reaction. It may be determined by any of three important methods (*a*) e.m.f. measurements (*b*) equilibrium data, and (*c*) from purely thermal data by means of the Third Law of thermodynamics.

Because ΔG is a measure of the capacity of a system for doing work it enables one to decide whether or not a reaction may occur spontaneously. Spontaneous reaction can occur only if there is a *decrease* in free energy (such reactions are termed *exergonic*); if there is an increase in free energy, then work must be put into the system to bring about the change. This is an *endergonic* reaction. By convention, a decrease in free energy is denoted by a negative sign and an increase by a positive sign. Hence if the free energy change for a reaction is $-5{,}000$ calories the reaction may occur spontaneously and there is a decrease in G of 5,000 calories. It does not automatically follow, however, that because a given reaction has a high negative value for ΔG the reaction takes place at a measurable rate. ΔG measures only the difference in free energy between the initial and final states of the reaction and does not give any information about the rate of reaction. To bring about appreciable reaction a catalyst may be necessary.

The free energy change of a reversible reaction is related to the equilibrium constant of the reaction (see p. 64) by the equation

$$\Delta G^\circ = -RT \ln K \quad . \quad . \quad . \quad (3.9)$$

where ΔG° is the *standard free energy change* and K the equilibrium constant. ΔG° signifies that the free energy change refers to the reaction when all the reactants and products are in their standard states. The standard state is a convenient reference condition in which the activities are arbitrarily defined as unity for pure liquids or solids, gases at 1 atmosphere, and compounds in

solution at approximately 1 M concentration[1] at a given temperature, usually 25°. It follows, therefore, that ΔG° is a constant for any given reaction. Values of ΔG° are additive: i.e. if such values are known for two reactions, that of a third may be calculated by methods the same as those applied to heats of reaction when the Law of Hess is used. The standard free energy ΔG° must not be confused with the free energy ΔG. They are related for the reaction $A + B \rightleftharpoons C + D$ by the equation

$$\Delta G = \Delta G^\circ + RT \ln \frac{[C][D]}{[A][B]} \quad . \quad . \quad (3.10)$$

where [A] and [B] are the initial concentrations of the reactants and [C] and [D] the final concentrations of the products. It is ΔG and not ΔG° which determines whether or not spontaneous reaction may occur. This distinction has not always been observed in the biochemical literature. ΔG° is, however, the value always tabulated for a given reaction because it is a defined quantity, whereas ΔG can have any value depending on the conditions as implied by equation 3.10. Unlike ΔG, to a very good first approximation ΔH is not dependent on the state or concentration of the reactants and hence may be equated with ΔH°.

Special mention must be made of the value taken for the concentration of water when standard free energy values are being calculated from equilibrium data. As a pure liquid its activity in the standard state may be taken as unity, but the actual molal concentration is $1000/18 = 55{\cdot}55$ in dilute aqueous solutions. Hence care must be exercised to assign the same value to water as has been used in defining ΔG°; otherwise a difference of 2,475 calories will arise at 37°. In calculating equilibrium constants, the activity of water, either in the pure state or in dilute solution, is usually taken as unity.

When a reversible system is at equilibrium, the free energy change is zero, i.e. $\Delta G = 0$, and therefore

$$\Delta H = T\Delta S.$$

[1] Strictly speaking the standard state for a solute is one of unit *activity*, the activity scale being so chosen that the ratio of activity to concentration (either molar or molal) tends to unity as the concentration approaches zero. Hence a solution of unit activity will not, in general, be exactly 1 molar (or molal). In thermodynamics the concentration is usually taken as molal, since it is then independent of temperature. For biochemical work in aqueous media the use of molar concentrations does not introduce appreciable error.

The entropy of the system and its surroundings is at a maximum and the system is in its most probable state. Sometimes an endergonic reaction, which will not of itself proceed because of the increase in free energy involved, can be made to do so by coupling it with an exergonic reaction. For this to occur there must be an intermediate common to both reactions. Consider the following three reactions:

1.	$A + B \rightleftharpoons C + D$	ΔG°_1 small positive (endergonic),
2.	$D + L \rightleftharpoons M + N$	ΔG°_2 large negative (exergonic),
3.	$A + B + L \rightleftharpoons C + M + N$	ΔG°_3 moderately negative (exergonic).

Compound D, formed in reaction 1, is a reactant in reaction 2, and the overall reaction then becomes 3, which is moderately exergonic ($\Delta G^\circ_1 + \Delta G^\circ_2$) and hence compound C will be formed from A and B. This may be regarded as an example of the principle of Le Chatelier whereby a reaction is made to proceed in the direction of completion by removal of one of the products. In this case D is removed by coupling it to a second reaction.

In living organisms many examples of coupled reactions occur. Of particular importance are reactions involving the so-called 'energy-rich' phosphate bonds. The hydrolysis of most phosphate esters results in a free energy change of approximately $-2{,}000$ calories, whereas the hydrolysis of compounds such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine phosphate and arginine phosphate gives a free energy change of approximately $-10{,}000$ to $-16{,}000$ calories.[1] Compounds which liberate this greater amount of energy on hydrolysis are termed 'energy-rich' and their importance lies in the fact that free energy liberated in metabolic processes may be 'trapped' or stored in these compounds. For example, ADP can be converted to ATP by the addition of an energy-rich phosphate bond, and the free energy thereby conferred can be transferred subsequently to other compounds or utilized to perform work. In muscle, for instance, the contractile protein myosin catalyses the hydrolysis of ATP to

[1] See GUTFREUND, (1954), *Ann. Rep. Chem. Soc.*, **51**, 295, for recent assessments of these values.

ADP; the energy liberated is used for the contraction process and mechanical work is done. ATP is regenerated from ADP by the action of phosphocreatine and thus a cyclic process operates:

$$\text{ATP} \xrightarrow{\text{myosin}} \text{ADP} + \text{free energy}$$

$$\text{ADP} + \text{phosphocreatine} \rightarrow \text{ATP} + \text{creatine}$$

The transference of a phosphate group and free energy is achieved in this manner. Creatine is subsequently rephosphorylated in readiness for further use. Phosphocreatine acts as a 'storehouse' of energy when the muscle is not active and its energy reserves are drawn upon during contraction.[1]

The generation of energy-rich phosphate bonds occurs in two major metabolic sequences, the glycolytic process and the tricarboxylic acid cycle, both of which proceed in a series of stages releasing graded amounts of energy. The overall liberation of free energy in the oxidation of glucose or pyruvic acid thereby occurs gradually, not suddenly nor in great amount. The organism is enabled to 'harness' the available energy as energy-rich phosphate bonds.

A word of caution is necessary here about the use of the term 'energy-rich phosphate bond' since some confusion of thought has arisen in biochemical circles from its use. The term is somewhat misleading, for it refers not to bond energy as defined by chemists but to the free energy resulting from hydrolysis of the phosphate compound. Bond energy in its chemical (and correct) sense is the energy which must be absorbed to break a bond of a gaseous molecule with the production of neutral gaseous atoms or radicals. A compound containing an energy-rich bond is simply a highly reactive, and hence relatively unstable compound the hydrolysis of which releases appreciable amounts of free energy. Really it is the molecule which is energy-rich rather than the bond broken; but, when the bond *has* been broken, the resulting molecule is more stable, i.e. less rich in energy. When viewed in this light, the energy-rich compounds fall into true perspective and can be classified along with (to use the same terminology) the

[1] But see FLECKENSTEIN, JANKE, DAVIES & KREBS (1954), *Nature*, **174**, 1081, and MOMMAERTS, *ibid.*, 1083, for recent work concerning the role of ATP in muscle contraction.

energy-rich compounds of organic chemistry, such as acetyl chloride and acetic anhydride.

The Third Law (Nernst Heat Theorem).—This is stated as, 'The entropy of a pure crystallized substance is zero at absolute zero.' Consequently, by determining the entropy increase of a substance as its temperature is raised from the absolute zero to any given temperature, the actual entropy of the substance at that temperature is obtained. Data for such reactions are obtained from measurements of specific heats and of latent heats associated with changes of state occurring as the substance is cooled from, say, 25° C. to the absolute zero. If the standard entropies of all the compounds in a reaction have been separately evaluated in this way, the change in entropy that occurs when they react can be calculated. Consider the reaction

$$A + B \rightarrow C + D.$$

The entropy change is

$$\Delta S = S_C + S_D - (S_A + S_B),$$

and, knowing ΔS, the free energy change for the reaction can be obtained from equation 3.8.

Parks and Huffman (1932) have compiled tables of entropies of compounds, and their studies reveal a relationship between chemical constitution and entropy. This is of value for the empirical calculation of entropies of unknown substances. The following examples illustrate the use of these entropy 'rules'.

All the data refer to 25°.

For each H atom in the molecule S increases by 11·3 e.u. (entropy units).

For each C atom in the molecule S decreases by 13·4 e.u.

The entropy contribution of oxygen is dependent on the type of linkage. Thus:

For each terminal O atom, as in $-OH$ of $-COOH$, S increases by 0·9 e.u.

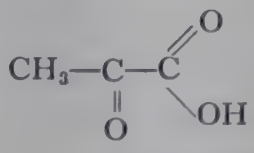
For each secondary O atom, as in $-OH$ of $-CHOH$, S decreases by 4·6 e.u.

For each carbonyl O atom, as in $=C=O$ of $-COOH$, S increases by 24·4 e.u.

Consequently the type of oxygen bond in a molecule plays an

important role in determining the entropy of a compound, and the large value of the carbonyl oxygen may well be a predominant factor in the entropy value.

Example 3.2.—Use the Parks and Huffman rules to calculate the entropy of pyruvic acid at 25° C.



4 H atoms	$4 \times 11{\cdot}3 = +45{\cdot}2$
3 C atoms	$-3 \times 13{\cdot}4 = -40{\cdot}2$
1 terminal O atom	$0{\cdot}9 = +\ 0{\cdot}9$
2 carbonyl O atoms	$2 \times 24{\cdot}4 = +48{\cdot}8$
Total = $S°_{298}$(pyruvic acid)	$= +54{\cdot}7$

Although the application of these empirical rules has been of great value when the entropy of a compound was required but thermal data were not available, it will be appreciated that thermodynamic quantities obtained more directly are greatly to be preferred. The very fact that modifications in structure profoundly affect entropy is an indication of the uncertainty that may be introduced if this quantity is calculated for an 'unknown' compound by the application of empirical rules.

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PROBLEMS

Note.—A study of Example 4.1 may assist the solution of some of these problems. Further problems of a similar nature will be found in Chapter IV.

3.1. The change in internal energy when solid palmitic acid is combusted in a bomb calorimeter at 20° is −2384·1 kcal. The equation for the reaction is:

$$C_{16}H_{32}O_{2(S)} + 23O_{2(G)} \rightarrow 16CO_{2(G)} + 16H_2O_{(L)}\ .$$

Determine the heat content change for the reaction.

3.2. The heat of combustion of stearic acid (solid) at 15° and 1 atmosphere pressure is −2698·0 kcal. What is the change in internal energy associated with the reaction?

3.3. The heat of combustion ($\triangle$H) of liquid ethanol to gaseous carbon dioxide and liquid water is −325·70 kcal. per mole at 15°. Calculate the internal energy change for the reaction.

The heats of formation of gaseous carbon dioxide and liquid water are −94·45 and −68·37 kcal. respectively at 15°. Determine the heat of formation of ethanol from its elements at this temperature.

3.4. At 25° and 1 atmosphere pressure the standard free energy of formation of solid α-D-glucose is −215,800 calories. The standard free energies of formation of gaseous carbon dioxide and liquid water are −94,450 and −56,690 calories respectively under similar conditions. Find the standard free energy of combustion of glucose to carbon dioxide and water at this temperature and pressure.

3.5. The standard free energy of formation of aqueous divalent succinate ion is −165,090 calories at 25° and 1 atmosphere. If the standard free energy of the reaction

$$\text{Fumarate}'' \text{ (aq.)} + H_2 \rightarrow \text{succinate}'' \text{ (aq.)}$$

is −20,470 calories under the same conditions, calculate the standard free energy of formation of aqueous divalent fumarate ion at 25°.

3.6. The standard free energies of formation of aqueous solutions of monovalent L-cysteine anion and divalent L-cystine anion are −70,270 and −137,190 calories respectively. What is the standard free energy change for the reduction of cystine to cysteine? The values refer to 25° and 1 atmosphere pressure.

3.7. The most general method for obtaining heats of reaction is by the determination of heats of combustion of the reactants and products. Recent attention has been focused on the direct calorimetric determination of the heat of reaction where this is possible. The following data illustrate both experimental methods.

Huffman and Fox determined the heats of combustion of solid α-D-glucose and β-D-glucose to gaseous carbon dioxide and liquid water at 25° and 1 atmosphere. The values obtained were, respectively, −669·58 and −671·08 kcal. per mole. Determine the heat of reaction for the mutarotation process.

Sturtevant has made direct calorimetric measurements of the heats of mutarotation in solution and the heats of solution of the two forms of glucose. He obtained the following results at 298° K.:

α-D-glucose (S) → α-D-glucose (aq.)	$\Delta H =$ 10,716 joules per mole.
α-D-glucose (aq.) → β-D-glucose(aq).	$\Delta H =$ −1,162 joules per mole.
β-D-glucose (aq.) → β-D-glucose (S)	$\Delta H =$ −4,680 joules per mole.

Calculate the heat of mutarotation of solid α-D-glucose to β-D-glucose. Compare this value with that obtained by the former method and express the difference as a percentage of the value obtained by the direct measurement, and also as a percentage of the average heat of combustion of the two glucose isomers. Comment on the accuracy of heats of reaction obtained by the heat of combustion method.

(Data from HUFFMAN & FOX (1938), *J. Amer. chem. Soc.*, **60**, 1400, and STURTEVANT (1941), *J. phys. Chem.*, **45**, 127.)

3.8. The heats of combustion of glucose, pyruvic acid and ethanol at 18° and 1 atmosphere are, respectively, −674·00, −279·10 and −326·66 kcal. per mole. What quantity of heat is evolved in the formation of 1 mole of (*a*) pyruvic acid (*b*) ethanol by the fermentation of glucose at 18° and 1 atmosphere? Neglect heats of dilution, etc.

3.9. The heat of combustion of solid anhydrous citric acid is −475·0 kcal. and that of solid malic acid −320·1 kcal. at 15° and 1 atmosphere pressure. Calculate the heat of reaction for the conversion of citric acid to malic acid at 15° and 1 atmosphere. The heats of formation of liquid water and gaseous carbon dioxide at the same temperature and pressure are −68·37 and −94·45 kcal. respectively.

3.10. Calculate the heat of reaction of the formic hydrogenlyase system of *Escherichia coli:*

$$H_2 + HCO_3' \rightleftharpoons HCOO' + H_2O$$

from the following thermal data, all of which applies to 298° K.

$$HCOOH\ (L)\ \triangle H = -99{,}750\ \text{cal.}$$
$$CO_2\ (G)\ \triangle H = -94{,}240\ \text{cal.}$$
$$H_2O\ (L)\ \triangle H = -68{,}310\ \text{cal.}$$
$$CO_2\ (G) \rightleftharpoons CO_2\ (\text{sat. aq.}) + 4844\ \text{cal.}$$
$$H_2CO_3 \rightleftharpoons H^+ + HCO_3' - 2075\ \text{cal.}$$
$$HCOOH\ (L) \rightleftharpoons HCOOH\ (\text{aq.}) + 100\ \text{cal.}$$
$$HCOOH\ (\text{aq.}) \rightleftharpoons H^+ + HCOO' + 13\ \text{cal.}$$

(Data quoted by Woods (1936), *Biochem. J.*, **30**, 515.)

3.11. From the entropy data of Parks and Huffman given on p. 59, calculate the entropy at 25° of the following compounds of biochemical interest: (*a*) lactic acid, (*b*) malic acid, (*c*) α-ketoglutaric acid, (*d*) oxaloacetic acid, (*e*) acetylmethylcarbinol.

3.12. The oxidation of phosphoglyceraldehyde to phosphoglyceric acid involves a free energy change of −29,000 cal. Under biological conditions this reaction is achieved via the formation of phosphoglycerylphosphate and requires the participation of diphosphopyridine nucleotide (DPN) as coenzyme, and also inorganic phosphate.

The reduction of DPN, viz.

$$DPN^+ + 2H \rightleftharpoons DPNH + H^+,$$

has $\triangle G = 13{,}000$ cal. and the overall reaction

$$\text{Phosphoglyceraldehyde} + \text{phosphate} + DPN^+ \rightarrow \text{phosphoglycerylphosphate} + DPNH + H^+$$

has $\triangle G = -1{,}000$ cal.

From these data, what do you deduce with regard to the nature of phosphoglycerylphosphate? State how phosphoglycerylphosphate is converted biologically into phosphoglyceric acid and indicate the value of this mechanism to the organism.

What would happen if phosphate were replaced by arsenate in the oxidation of phosphoglyceraldehyde?

The oxidation of lactic acid to pyruvic acid has $\triangle G = -9{,}000$ cal. Is this compatible with the triosephosphate dehydrogenase and lactic dehydrogenase systems being 'coenzyme-linked'?

(Glasgow Honours Course Finals, 1951.)

CHAPTER IV

EQUILIBRIA

Consider the reversible chemical reaction

$$A + B \rightleftharpoons C + D.$$

The rate of the forward reaction is $k_1(A)(B)$ and the rate of the reverse reaction is $k_2(C)(D)$, where the concentrations (more accurately active masses) of the reactants are represented by (A), (B), (C) and (D). At equilibrium the rates of the forward and reverse reactions will be equal so that, employing equilibrium concentrations,

$$k_1(A)(B) = k_2(C)(D) \quad . \quad . \quad . \quad (4.1)$$

and

$$\frac{(C)(D)}{(A)(B)} = \frac{k_1}{k_2} = K \quad . \quad . \quad . \quad (4.2)$$

where K is the *equilibrium constant* of the reaction. Note the convention that the concentrations of the products of the reaction, i.e. right-hand side of the chemical equation, are put in the numerator. This equation represents the Law of Mass Action, first enunciated by Guldberg and Waage in 1864. From the above relation it follows that, irrespective of the initial concentrations of A, B, C and D, at equilibrium the concentrations of all molecular species are related to one another in such a way that equation 4.2 holds. Consequently, if K is known, the position of equilibrium may be calculated for any initial concentrations of the reacting substances.

The example quoted is a simple case where one molecule of each substance reacts, but frequently more complicated reactions are encountered. The general case of a reaction such as:

$$aA + bB + cC + \cdots \rightleftharpoons pP + qQ + rR + \cdots$$

where a, b, etc., represent the respective numbers of molecules of A, B, etc., reacting, leads to the following formulation of the equilibrium constant

$$K = \frac{(P)_e^p(Q)_e^q(R)_e^r \cdots}{(A)_e^a(B)_e^b(C)_e^c \cdots} \quad . \quad . \quad . \quad (4.3)$$

It will be noted that the equilibrium concentration of each substance is raised to the power of the number of its molecules participating in the reaction.

The Law of Mass Action may also be derived thermodynamically and the relationship of the equilibrium constant to the free energy change expressed as :

$$\Delta G = -RT \ln K + RT \ln \frac{(\mathrm{P})^p(\mathrm{Q})^q(\mathrm{R})^r \ldots}{(\mathrm{A})^a(\mathrm{B})^b(\mathrm{C})^c \ldots} \quad . \quad (4.4)$$

This may be contracted by the use of algebraic notation to

$$\Delta G = -RT \ln K + RT \ \Sigma a \ln (\mathrm{A}) \quad . \quad (4.5)$$

where the second right-hand term represents the sum of the logarithms of the concentration of each reacting substance multiplied by the number of molecules of the substance, with correct sign, i.e. all numerator concentrations are positive and all denominator concentrations negative. If all the substances are in their standard states of unit activity, the last term becomes $RT \ln 1$ which is zero and hence equation 4.5 reduces to

$$\Delta G^\circ = -RT \ln K \quad . \quad . \quad . \quad (4.6)$$

where ΔG° is the *standard free energy change* for the reaction. It follows, therefore, that

$$\Delta G = \Delta G^\circ + RT \ \Sigma a \ln (\mathrm{A}) \quad . \quad . \quad (4.7)$$

Consequently, the standard free energy change of a reaction may be calculated from the equilibrium constant by means of equation 4.6 and then used in equation 4.7 to obtain the free energy change for the reaction with any arbitrary concentrations. Once obtained, standard free energies have the valuable property of being additive like heats of reaction and so enable the value for a particular reaction to be calculated from those previously established.

In many cases when it may prove difficult or even impossible to measure an equilibrium constant directly, recourse to calculation by indirect methods is necessary. Equation 4.6 is the basis of all such calculations. For example, ΔG° for several partial reactions, the summation of which is the reaction in question, may be added together to give ΔG° for this reaction and hence K. ΔG° for the partial reactions may be obtained either by equilibrium or by e.m.f. measurements. Sometimes the values of the standard free

energy of formation of compounds from their elements can be found in suitable reference books (Landolt-Börnstein, Parks and Huffman) and ΔG° for the total reaction obtained by summation. It is important to note that the value of the standard free energy of formation (usually denoted by ΔG_f°) employed must refer to the state which obtains in the reaction. For instance, if the compound is reacting in solution ΔG_f° must refer to the solution of appropriate activity and not simply to ΔG_f° of the solid compound. Since ΔG_f° of a saturated solution is equal to that of the solid compound, this value must be added to the standard free energy of dilution of a saturated solution to a solution of the given activity. Furthermore, should the compound be present in solution as an ion, the standard free energy of ionization must also be added. For a substance dissolved in water the standard state is that at which the concentration of the solute is approximately 1 molar (see p. 56), and the following relationship holds for the transference of a substance from the standard to another state by alteration of either concentration, pressure or temperature

$$\Delta G = \Delta G^\circ + RT \ln a,$$

where a is the activity.

Example 4.1.—Calculate the equilibrium constant for the formation of the dipeptide glycylglycine from two molecules of glycine at 37·5° C. The standard free energies of formation from their elements to the standard state in aqueous solution (1 M activity, except for water, which is 1 mole fraction) are, at 37·5° C., respectively: glycine, −87,710 calories; glycylglycine, −115,630 calories; water, −56,200 calories.

The reaction is

$$2 \text{ glycine} \rightleftharpoons \text{glycylglycine} + H_2O.$$

Hence $\Delta G^\circ = \Delta G_f^\circ$ (glycylglycine) + $\Delta G_f^\circ(H_2O) - 2\Delta G_f^\circ$ (glycine)

$= -115630 - 56200 - 2(-87710)$

$= -171830 + 175420$

$= \underline{3{,}590 \text{ calories.}}$

Now $\Delta G^\circ = -RT \ln K$

$\therefore 3590 = -2{\cdot}303 \times 1{\cdot}987 \times 310{\cdot}5 \log K$

$\log K = -2{\cdot}526 = \bar{3}{\cdot}474$

$\therefore \underline{K = 0{\cdot}00298.}$

Consider now the reversible reaction

$$aA + bB \rightleftharpoons cC + dD.$$

The change in free energy for this reaction is expressed by equation 4.5, which becomes

$$\Delta G = -RT \ln K + RT \ln \frac{(C)^c(D)^d}{(A)^a(B)^b}.$$

At thermodynamic equilibrium in a reversible reaction ΔG is zero and since $\Delta G = \Delta H - T\Delta S$ it follows that, at equilibrium, the capacity of the system to do work is at a minimum and the entropy is at a maximum.

Example 4.2.—The following data were obtained in an experiment to determine the equilibrium constant of the reaction

$$\textit{iso}\text{-propanol} + DPN^+ \rightleftharpoons \text{acetone} + DPNH + H^+,$$

which is carried out by crystalline yeast alcohol dehydrogenase.

	*p*H	*Acetone* (M × 10^2)	Iso-*propanol* (M × 10^2)	*DPNH* (M × 10^5)	*DPN*$^+$ (M × 10^5)
(*a*)	8·78	19·5	3·92	4·74	5·41
(*b*)	7·28	1·54	8·49	4·51	6·00
(*c*)	7·18	1·51	8·33	3·43	5·72

The values given are for the concentrations of the compounds at equilibrium in experiments carried out at 25° C.

Determine whether or not the equilibrium constant is influenced by pH and also the standard free energy change for the reaction. The activity coefficients of all the reactants may be taken as unity.

(After BURTON & WILSON (1953), *Biochem. J.*, **54**, 86.)

The activities are equal to the concentrations of the reactants since all activity coefficients are unity and the equilibrium constant is therefore given by the equation

$$K = \frac{(\text{acetone})(DPNH)(H^+)}{(\textit{iso}\text{-propanol})(DPN^+)} = \frac{(\text{acetone})(DPNH)}{(\textit{iso}\text{-propanol})(DPN^+)} \times \frac{1}{\text{antilog pH}}$$

since $(H^+) = \dfrac{1}{\text{antilog pH}}$.

Hence in experiment (*a*) at pH 8·78

$$K = \frac{19{\cdot}5 \times 10^{-2} \times 4{\cdot}74 \times 10^{-5}}{3{\cdot}92 \times 10^{-2} \times 5{\cdot}41 \times 10^{-5}} \times \frac{1}{\text{antilog } 8{\cdot}78}$$

$$= \frac{19{\cdot}5 \times 4{\cdot}74}{3{\cdot}92 \times 5{\cdot}41 \times 6{\cdot}026 \times 10^8} = 7{\cdot}23 \times 10^{-9} \text{ M}.$$

In experiment (*b*) at pH 7·28

$$K = \frac{1{\cdot}54 \times 4{\cdot}51}{8{\cdot}49 \times 6{\cdot}00} \times \frac{1}{\text{antilog } 7{\cdot}28} = 7{\cdot}16 \times 10^{-9} \text{ M}.$$

and in experiment (*c*) at pH 7·18

$$K = \frac{1{\cdot}51 \times 3{\cdot}43}{8{\cdot}33 \times 5{\cdot}72} \times \frac{1}{\text{antilog } 7{\cdot}18} = 7{\cdot}20 \times 10^{-9} \text{ M}.$$

From these results it is obvious that the value of K has not been appreciably affected by changing the pH value from 8·78 to 7·18. The average value of the

equilibrium constant from the above three values is $7{\cdot}196 \times 10^{-9}$ M, and this may now be used to calculate the standard free energy change of the reaction

$$\begin{aligned}\Delta G^\circ &= -RT \ln K = -2{\cdot}303\ RT \log K \\ &= -2{\cdot}303 \times 1{\cdot}987 \times 298 \log 7{\cdot}196 \times 10^{-9} \\ &= -2{\cdot}303 \times 1{\cdot}987 \times 298\ (\bar{9}{\cdot}8571 = -8{\cdot}1429) \\ &= \underline{11{,}105 \text{ calories.}}\end{aligned}$$

Effect of Temperature on the Equilibrium Constant.

Van't Hoff deduced the following relationship for the variation of the equilibrium constant with absolute temperature

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2} \quad . \quad . \quad . \quad (4.8)$$

where ΔH is the heat of reaction at constant pressure. This is known as the *van't Hoff equation* or *isochore*. Integrating equation 4.8

$$\ln \frac{K_2}{K_1} = \frac{-\Delta H}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \quad . \quad . \quad (4.9)$$

$$\ln \frac{K_2}{K_1} = \frac{\Delta H}{R}\left(\frac{T_2 - T_1}{T_1 T_2}\right) \quad . \quad . \quad (4.9a)$$

where K_1 and K_2 are the values of the equilibrium constant at temperatures T_1 and T_2. Thus if $\log K$ is plotted against $\frac{1}{T}$, a straight line of slope $\frac{-\Delta H}{2{\cdot}303R}$ will be obtained, enabling ΔH for the reaction to be evaluated.

Activities and Concentrations.

In order that a distinction may be made between equilibrium constants calculated from the equilibrium activities of the reactants and those calculated from equilibrium concentrations, these constants are often denoted in the literature by K_a and K_c respectively. For the reaction

$$A + B \rightleftharpoons C + D$$

they are related in the following manner:

$$K_a = \frac{f_C[C] f_D[D]}{f_A[A]\, f_B[B]} \quad \text{and} \quad K_c = \frac{[C][D]}{[A][B]}$$

whence $$K_a = K_c \frac{f_C f_D}{f_A f_B}$$

Thus K_a can be obtained from K_c provided that the activity coefficients of all reactants are known.

Quite often in biochemical work the equilibrium constant of a reaction involving hydrogen ions is calculated without considering the pH, e.g. in reactions involving DPN, such as:

$$C_2H_5OH + DPN^+ \rightleftharpoons CH_3CHO + DPNH + H^+$$

the equilibrium constant may be written as:

$$K_{app} = \frac{(CH_3CHO)(DPNH)}{(C_2H_5OH)(DPN^+)}$$

and the hydrogen ion term omitted from the numerator. The constant obtained in this manner is termed an *apparent equilibrium constant*, denoted by K_{app}, to distinguish it from the true equilibrium constant calculated by inclusion of the hydrogen ion activity. Examples of this usage will be found in Problems 4.11 and 4.12.

REFERENCES

LANDOLT-BÖRNSTEIN (1923-36). *Physikalisch-chemische Tabellen*, 5th ed. Berlin: Springer-Verlag.

PARKS, G. S., & HUFFMAN, H. M. (1932). *The Free Energies of Some Organic Compounds*. New York: Chemical Catalogue Company.

PROBLEMS

Note.—In all the problems in this chapter it may be assumed, unless stated to the contrary, that the activity coefficients are unity.

The formulation of the reactions involving diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) is that of the authors of the papers quoted. This is the reason for lack of uniformity in the equations representing nucleotide reduction.

4.1. Calculate the equilibrium constant of the hydrolysis of DL-alanylglycine by a suitable peptidase at 37·5° C.

ΔG_f° values for the compounds involved, in their standard state in aqueous solution are, at 37·5° C., as follows: alanylglycine, −114,680 cal.; alanine, −87,300 cal.; glycine, −87,710 cal.; water, −56,200 cal.

4.2. Calculate the standard free energy of formation of the hippurate ion if the equilibrium constant for the formation of hippurate from benzoate and glycine is 0·0142 at 37·5° C.

ΔG_f° values at 37·5° C. are: benzoate ion, −49,710 cal.; glycine, −87,710 cal. for the standard states in aqueous solution, and for water, −56,200 cal.

4.3. Determine the standard free energy of formation of benzoylglycylglycine in aqueous solution at 25° C. from the following data. The equilibrium constant of the formation of benzoylglycylglycine from benzoate ion and glycylglycine is 0·1564 at 25° C. and, at the same temperature, the standard free energy change for the condensation of two glycine molecules to yield glycylglycine is 3,530 calories. Standard free energies of formation of benzoate ion and glycine at 25° C. are, respectively, −51,175 cal. and −89,140 cal. for the standard states in aqueous solution. ΔG_f° for water at 25° C. is −56,690 cal.

4.4. Calculate the equilibrium constants at 38° C. for the following reactions:

(*a*) oxaloacetate″ + $H_2O \rightleftharpoons$ pyruvate′ + HCO_3'.

(*b*) fumarate″ + $2H_2O \rightleftharpoons$ lactate′ + HCO_3'.

(*c*) fumarate″ + $H_2O \rightleftharpoons$ malate″.

The values for the standard free energy of formation of these compounds at 38° C. are, according to Borsook, as follows: pyruvate′, −106,460 cal.; lactate′, −117,960 cal.; HCO_3' −139,200 cal.; malate″, −199,430 cal.; H_2O, −56,200 cal.; fumarate″, −142,525 cal.; oxaloacetate″, −184,210 cal. These values apply to the standard state in aqueous solution.

(Data from EVANS, VENNESLAND & SLOTIN (1943), *J. biol. Chem.*, **147**, 771.)

4.5. Calculate the equilibrium constant at 25° C. of the 'aldehyde mutase' reaction, which may be expressed as:

$$2CH_3CHO + H_2O \rightleftharpoons CH_3COOH + CH_3CH_2OH.$$

The standard free energies of formation of the respective reactants at 25° C. are: acetaldehyde aq., −33,000 cal.; acetic acid aq., −96,210 cal.; ethanol aq., −43,850 cal. and water (liquid), −56,560 cal.

What concentration of acetaldehyde will be in equilibrium with 0·1 M acetic acid and 0·1 M ethanol?

The assumption has been made above that the acid is not appreciably dissociated; the reaction is usually carried out in a bicarbonate-CO_2 buffer system and may be represented as:

$$2CH_3CHO \text{ aq.} + HCO_3' \rightleftharpoons CH_3COO' + CH_3CH_2OH \text{ aq.} + CO_2(g).$$

This formulation takes account of the ionization and neutralization of the acid. ΔG_f° at 25° C. for HCO_3' is −140,000 cal.; for CH_3COO' is −89,720 cal.; and for gaseous CO_2 is −94,100 cal.

Calculate the equilibrium constant of this reaction and the concentration of acetaldehyde in equilibrium with 1 M acetate and ethanol if the bicarbonate concentration is 0·03 M and the partial pressure of CO_2 is 0·05 atmosphere.

(After DIXON (1939), *Ergeb. Enzymforsch.*, **8**, 217.)

Note.—This reaction is now known to consist of two coupled reactions, alcohol dehydrogenase and aldehyde dehydrogenase, viz.

$$CH_3CHO + 2DPNH \rightleftharpoons CH_3CH_2OH + 2DPN$$
$$CH_3CHO + H_2O + 2DPN \rightleftharpoons CH_3COOH + 2DPNH$$
$$\overline{2CH_3CHO + H_2O \rightleftharpoons CH_3CH_2OH + CH_3COOH}$$

(RACKER (1949), *J. biol. Chem.*, **177**, 883.)

4.6. The enzyme phosphoglucomutase catalyses the interconversion of glucose-1-phosphate and glucose-6-phosphate. At 38° C., the equilibrium mixture contains 5·4 per cent. of the 1-phosphate. Calculate the equilibrium constant and the standard free energy change of the reaction.

4.7. The triosephosphate isomerase of muscle catalyses the reversible conversion of glyceraldehyde phosphate to dihydroxyacetone phosphate.

At 37° C. the standard free energy change of the reaction is −2,000 calories. Calculate the equilibrium concentrations of the two reactants.

4.8. The aspartase enzyme of *Escherichia coli* catalyses the reversible reaction:

$$\text{Fumarate}'' + NH_3 \rightleftharpoons \text{L-aspartate}''.$$

Starting with fumarate and ammonia concentrations of 0·1 M, 28 per cent. of the original ammonia remained at equilibrium when the reaction was carried out at 37° C. The activity coefficients for the compounds involved are $f_{fum.}$0·7, $f_{asp.}$0·7 and f_{NH_3}0·22. Determine the standard free energy change for the reaction.

(Data from BORSOOK & HUFFMAN (1933), *J. biol. Chem.*, **99**, 663.)

4.9. The following data were obtained in an experiment to investigate the effect of temperature on the equilibrium constant of the fumarase system which catalyses the reaction

$$\text{Fumarate}'' + H_2O \rightleftharpoons \text{Malate}''.$$

Temperature °C.	15	20	25	30	35	40	45	50
Equilibrium constant, K	4·786	4·467	4·074	3·631	3·311	3·090	2·754	2·399

Determine graphically the heat change for the hydration of fumarate and calculate also the standard free energy change of the reaction at 25° C. and 38° C.

(After SCOTT & POWELL (1948), *J. Amer. chem. Soc.*, **70**, 1104.)

4.10. The malic dehydrogenase enzyme from horse heart catalyses the reversible reaction

$$\text{L-malate}'' + DPN^+ \rightleftharpoons \text{oxaloacetate}'' + DPNH + H^+.$$

The equilibrium constant of this reaction was determined by following the change in optical density at 340 mμ in the initial 10 minutes; this enabled the concentration of reduced DPN to be obtained and that of oxaloacetate was assumed to be the same. The concentrations of L-malate and DPN^+ were obtained from the initial concentrations and those of the DPNH formed. These equilibrium figures are recorded below for experiments carried out at three different pH values and at 25°.

	pH	*DPNH* (=*oxaloacetate*) (M × 10^5)	*DPN*$^+$ (M × 10^5)	*L-Malate* (M × 10^3)
(*a*)	8·81	2·82	32·4	5·27
(*b*)	8·83	3·27	41·3	5·14
(*c*)	7·55	0·79	43·7	5·19

Calculate the equilibrium constant for each experiment and comment on the effect of pH on its value.

The activity coefficients may be assumed to be unity in every case. Determine the standard free energy change of the reaction.

(After BURTON & WILSON (1953), *Biochem. J.*, **54**, 86.)

4.11. The enzymatic synthesis of citric acid has been studied spectrophotometrically by coupling the condensation reaction (1) with the malic dehydrogenase system (2)

(1) $\text{Acetyl-CoA} + \text{oxaloacetate}'' + H_2O \rightleftharpoons \text{citrate}''' + \text{CoA} + H^+$.

(2) $\text{L-malate}'' + DPN^+ \rightleftharpoons \text{oxaloacetate}'' + DPNH + H^+$.

(3) Net reaction: $\text{L-malate}'' + \text{acetyl-CoA} + DPN^+ + H_2O \rightleftharpoons \text{citrate}''' + \text{CoA} + DPNH + 2H^+$.

In this way the reduction of DPN may be followed and the equilibrium constant of reaction (3) determined. This has been done by measuring the ratio of DPN to DPNH at varying ratios of malate to citrate with constant initial concentrations of acetyl-CoA and constant concentrations of condensing enzyme (enzyme (1)) and malic dehydrogenase (enzyme (2)).

The following data were obtained at pH 7·2 and 22°:

Equilibrium concentrations ($\times 10^3$M)					
Citrate	*CoA*	*DPNH*	*L-malate*	*Acetyl-CoA*	*DPN*$^+$
16·7	0·028	0·028	0·139	0·057	0·132
16·7	0·057	0·057	1·80	0·028	0·103
16·7	0·065	0·065	3·44	0·020	0·095
116·7	0·039	0·039	3·46	0·046	0·121

Calculate the apparent equilibrium constant of reaction (3) from this information. The concentration of water may be taken as 55·5 M. The apparent equilibrium constant of reaction (2) at pH 7·2 and 22° has been found to be $2{\cdot}33 \times 10^{-5}$. From this deduce the apparent equilibrium constant of the citrate synthesis reaction (reaction 1) at pH 7·2 and 22° and use this value to calculate the standard free energy change of the reaction.

(Data from STERN, OCHOA & LYNEN (1952), *J. biol. Chem.*, **198**, 313.)

4.12. The equilibrium constant of the 'malic' enzyme of wheat germ, which catalyses the reaction:

$$\text{Pyruvate}' + CO_2 + \text{TPNH} \rightleftharpoons \text{L-malate}'' + \text{TPN}^+$$

has been investigated. The change in absorption due to TPN was followed spectrophotometrically at 340 mμ at pH 7·3 and 22° C. The gas phase employed was nitrogen containing CO_2 to various concentrations and concentrations of L-malic, pyruvic acid and TPN in the solutions were determined enzymatically. The values of (H_2CO_3) below give the total concentration in solution of free CO_2 and may be used as such in the calculation of the equilibrium constants. The following data were obtained:

Initial concentration of reactants moles/litre $\times 10^3$				*Concentration of reactants at equilibrium*, moles/litre $\times 10^3$		
TPN$^+$	*L-malate*	*Pyruvate*	(H_2CO_3)	*TPNH*	*TPN*$^+$	*L-malate*
0·0530	0·657	32·6	1·8	0·0195	0·0335	0·637
0·0530	0·986	16·3	1·8	0·0357	0·0187	0·952
0·1060	0·657	32·6	1·8	0·0371	0·0689	0·620
0·0530	0·657	16·3	3·6	0·0178	0·0352	0·620
0·0530	1·32	16·3	7·2	0·0170	0·0360	1·303

From these data determine the apparent equilibrium constants of the individual reactions and obtain an average value for them.

Note.—Since the concentrations of pyruvate and CO_2 are large relative to those of TPN and L-malate, their variation in a given experiment may be neglected. Hence they may be assumed to be constant in the calculation of the individual equilibrium constants.

The equilibrium constant of the malic dehydrogenase reaction

$$\text{malate}'' + \text{DPN}^+ \rightleftharpoons \text{oxaloacetate}'' + \text{DPNH}$$

has been determined and a value of $2{\cdot}33 \times 10^{-5}$ obtained at pH 7·2 and 22° C. Assuming that the value of the equilibrium constants would be independent

of the pyridine nucleotide participating in the reaction, calculate the apparent equilibrium constant for the carboxylation of pyruvate, i.e.

$$\text{pyruvate}' + CO_2 \rightleftharpoons \text{oxaloacetate}''.$$

(Data from HARARY, KOREY & OCHOA (1953), *J. biol. Chem.*, **203**, 595.)

Note.—The authors of this work calculate the apparent equilibrium constants in accordance with the equations given above which omit the hydrogen ion term associated with the reduced form of the pyridine nucleotide (compare with Problem 4.11). As an additional exercise, calculate the equilibrium constants for the above reactions, taking into account the hydrogen ion term.

4.13. The synthetic action of alkaline intestinal phosphatase in the formation of glycerophosphate from glycerol and inorganic phosphate has been studied at 38° under appropriate conditions. The reaction mixtures were allowed to react for varying times and the final concentrations of glycerol and of ester formed were determined. It was found that practically all of the ester formed was of the α form. Data for four experiments carried out at pH 8·5 and for one at pH 5·8 are given below:

pH	*Time hours*	*Initial molar concentration*			*Final molar concentration*		
		Glycerol	*Phosphate*	H_2O	*Glycerol*	*Total ester*	*α-ester per cent.*
8·5	168	7·10	0·405	27·0	6·99	0·110	98
	144	1·70	0·480	48·8	1·677	0·0226	99
	700	11·19	0·0863	10·3	11·14	0·0552	87
	800	11·13	0·0863	10·3	11·13	0·0583	84
5·8	48	7·08	0·408	26·9	7·02	0·0610	99

Determine the equilibrium constant for the formation of total ester and the standard free energy change of the reaction at both pH values. The standard state of water should be taken as 55·5 M.

(After MEYERHOF & GREEN (1949), *J. biol. Chem.*, **178**, 655.)

4.14. The synthesis of formic acid from hydrogen and carbon dioxide by a reversal of the formic hydrogenlyase enzyme of *Escherichia coli* has been investigated and the equilibrium constant of the reaction:

$$H_2 + HCO_3' \rightleftharpoons H.COO' + H_2O$$

determined at two different temperatures. Experiments were carried out in Warburg manometers at pH 7·4 using formate concentrations covering the range 0·1 – 0·0125 M, a bicarbonate concentration of 0·025 M and a gas phase of 5 per cent. CO_2 in hydrogen. Initial rates of H_2 uptake or evolution were measured in order that the concentration of bicarbonate should not decrease appreciably during the experiment.

Figures obtained for the gas exchanges are given in the following table (controls have been subtracted from all the figures given).

Formate concentration M	*Hydrogen uptake or evolution* μl/hr.	
	25·1° C.	37·7° C.
0·10	+200	+296
0·05	+ 72	+140
0·025	− 25	+ 25
0·0125	− 93	− 46

The partial pressure of hydrogen at equilibrium was 0·916 and 0·884 atmospheres respectively for 25·1° and 37·7° (these values are corrected for atmospheric pressure and water vapour pressure).

(*a*) Determine graphically the concentration of formic acid present at equilibrium for the two temperatures and then use these values to calculate the equilibrium constants. The activity of hydrogen may be taken as its partial pressure and the activity of water as its mole fraction (0·988 under the experimental conditions employed). The activity coefficients of HCO_3' and H.COO′ may be taken as both equal to 0·762.

(*b*) Calculate the free energy change of the reaction.

(*c*) Calculate the heat of reaction.

(After Woods (1936), *Biochem. J.*, **30**, 515.)

4.15. The equilibrium constants of the aconitase system have been determined at 25° and 38°, when the following equilibrium concentrations of tricarboxylic acids were obtained at pH 7·4.

Temperature	*Equilibrium concentrations (per cent.)*		
	Iso-*citric acid*	cis-*aconitic acid*	*citric acid*
25	6·20	2·90	90·90
38	6·60	4·30	89·10

Determine the equilibrium constants from these data.

(After Krebs (1953), *Biochem. J.*, **54**, 78.)

4.16. The aspartic-glutamic transaminase system carries out the reaction

L-glutamate + oxaloacetate⇌α-ketoglutarate + L-aspartate

Krebs has determined the equilibrium concentrations of all reactants at 25° and pH 7·4 in a series of experiments in which the reaction was allowed to continue for 30, 40 and 50 minutes. Data from one such experiment are given below. Use this information to comment on the attainment of equilibrium in the time intervals investigated.

Period incubation (min.)	*Equilibrium concentrations (μl. substrate/ml. solution)*			
	Oxaloacetate	*Glutamate*	*α-Ketoglutarate*	*Aspartate*
30	111	121	298	307
40	106	120	289	298
50	108	123	297	305

(After Krebs (1953), *Biochem. J.*, **54**, 82.)

4.17. The equilibrium constant of the glutamate-alanine transaminase system has been determined by Krebs and has a value of 1·50 at 25° and pH 7·4.

i.e. L-glutamate + pyruvate⇌α-ketoglutarate + L-alanine ($K = 1{\cdot}50$)

If equal initial concentrations of L-glutamate and pyruvate of 430 μl. substrate per ml. are reacted under these conditions determine the equilibrium concentrations of all reactants.

(After Krebs (1953), *Biochem. J.*, **54**, 82.)

4.18. The determination of the equilibrium constant of the beef heart transhydrogenase reaction, which is

DPN + TPNH⇌TPN + DPNH

has been carried out and the following experimental data obtained when the reaction was allowed to continue for 40 minutes at 37° C.

pH	*Time (min.)*	*Concentration (μmoles/ml.)*			
		TPN	*DPNH*	*TPNH*	*DPN*
6·5	0			0·88	1·58
	40	0·66	0·60	0·31	0·99
6·5	0	1·33	1·18		
	40	0·83	0·56	0·45	0·52
6·0	0			0·96	1·57
	40	0·63	0·55	0·25	1·04
6·0	0	1·35	0·74		
	40	0·90	0·36	0·42	0·48
7·5	0			0·92	1·08
	40	0·56	0·60	0·42	0·58
7·5	0	0·98	1·08		
	40	0·68	0·63	0·53	0·50

Compare the equilibrium constants for the reaction in both directions at the various pH values and comment on the consistency of the values obtained.

(After KAPLAN, COLOWICK & NEUFELD (1953), *J. biol. Chem.*, **205**, 1.)

4.19. (*a*) The calcium ions in human serum combine with the protein(s) in a way which has been expressed by the following equation :

$$\text{Ca Prot} \rightleftharpoons \text{Ca}^{2+} + \text{Prot}''$$

The following analytical data are given by McLean and Hastings :

Concentrations expressed as m-moles/kg. H_2O

Total Protein	*Total Calcium*	*Free Ca^{2+}*
9·69	3·07	1·35
7·10	2·27	1·15
4·70	1·49	0·90
2·31	0·74	0·55

Use the mass law equation to calculate the dissociation constant, K, for each of the four sets of data.

(*b*) Does the agreement of the calculated dissociation constants in part (*a*) establish the correctness of the postulated reaction ? What restrictions do these data place on any alternative formulation of the reaction ?

(*c*) Suppose some calcium chloride is added to serum and the following values obtained soon afterwards :

Total Protein	. . .	9·36 m-moles/kg. H_2O
Total Calcium	. . .	4·47 ,,
Free Ca^{2+}	. . .	2·51 ,,

Does it appear that equilibrium has been reached ? If not, what will be the final concentration of each species ?

(*d*) If this final equilibrium mixture in part (*c*) is diluted with an equal volume of isotonic sodium chloride, will the resulting mixture be in equilibrium ? If not, in which direction will the reaction proceed ?

(*e*) The neutral salt calcium citrate, Ca_3Cit_2, has a primary dissociation which appears to go to completion :

$$Ca_3Cit_2 \rightleftharpoons Ca^{2+} + 2CaCit'$$

The secondary dissociation

$$CaCit' \rightleftharpoons Ca^{2+} + Cit'''$$

is found to have a dissociation constant of $6{\cdot}03 \times 10^{-4}$.

If 500 mg. of anhydrous sodium citrate, Na_3Cit, are dissolved in 1 litre of the equilibrium mixture of part (*c*), what will be the final concentration of free calcium ions and calcium protein complex in the resulting solution ? Neglect volume changes caused by the addition of the solid salt.

(Data from McLEAN & HASTINGS (1935), *J. biol. Chem.*, **108**, 285, and HASTINGS, McLEAN, EICHELBERGER, HALL & DACOSTA (1934), *J. biol. Chem.*, 107, 351. Harvard Medical Sciences 201 ab.)

CHAPTER V

REACTION KINETICS

THE velocity of a chemical reaction is dependent upon a variety of factors such as the concentrations of reacting molecules, the temperature and pressure, the presence or absence of catalysts, and the pH of the surrounding medium. The reaction velocity may be measured by following the rate of disappearance of a reactant or the rate of appearance of a product per unit time. To be of value, the time interval must be short and the measurement made in the initial stages of the reaction, otherwise considerable error may arise, especially with enzymic reactions where the enzyme may be poisoned by one of the products of the reaction. Usually the rate of reaction is a function of the concentration of one or more of the substances present. This is conveniently expressed in terms of the *order of the reaction.*

Here the difference between the order of a reaction and its *molecularity* must be emphasized. The former is the experimentally determined number of atoms or molecules the concentrations of which determine the observed kinetics, whereas the molecularity is the number of atoms or molecules that take part in the actual molecular mechanism; they are not necessarily the same. For instance, many first-order reactions involve more than one molecule and therefore are not necessarily unimolecular. (Note that in the following discussion concentrations are considered to be equivalent to the activities.)

Zero order reactions occur when the rate is entirely independent of the concentration of the reacting substance. This may happen in photochemical reactions and under certain circumstances in surface catalysis, such as enzymic reactions (see p. 97).

First order reactions take place at a rate proportional to the concentration of the reacting substance. Suppose a molecule A breaks down to give a product or products

$$A \rightarrow X, \text{ etc.}$$

The rate is

$$-\frac{d(A)}{dt} = k(A) \quad . \quad . \quad . \quad (5.1)$$

Integrating the expression and converting to $\log_{10}$

$$\log \frac{A_0}{A} = \frac{kt}{2{\cdot}303} \quad . \quad . \quad . \quad (5.2)$$

where A_0 is the initial concentration and A the concentration after time t. The *rate constant*, or *specific reaction rate*, k has the dimensions of reciprocal time and is usually expressed as sec.$^{-1}$ or min.$^{-1}$. Notice particularly that k is independent of the concentration units employed. For first order kinetics the plot of log A_0/A (or, more generally, log C where C is the concentration) against t gives a straight line of slope $k/2{\cdot}303$.

Many first order biochemical reactions are apparent or pseudo unimolecular reactions although the mechanism is more complex. For example, the rate of reaction may be equal to k(A)(B) where substance A disappears in the reaction but substance B does not. B, therefore, is a catalyst, the concentration of which remains constant throughout the reaction. Many enzyme reactions come within this category. Since (B) is constant, the velocity becomes

$$V = k'(A), \text{ where } k' = k(B) \quad . \quad . \quad (5.3)$$

i.e. first order although the mechanism is bimolecular.

Some important first order reactions, which are now of considerable interest in biochemistry, are radioactive decay processes (see Chapter XI). The wide application of radioactive elements as 'tracers' in biological experiments necessitates a thorough understanding of the decay processes by biochemists. The rate of decay is given by

$$-\frac{dn}{dt} = kn \quad . \quad . \quad . \quad . \quad (5.4)$$

where n is the number of radioactive atoms and k is the rate constant, in this case usually called the *decay constant*.

Integrating, $n = n_0 e^{-kt}$

$$kt = \ln \frac{n_0}{n} = 2{\cdot}303 \log \frac{n_0}{n} \quad . \quad . \quad . \quad (5.5)$$

where n_0 is the number of atoms at time $t = 0$ and n the number of atoms after time t. (Note the resemblance to the expression for the logarithmic growth of bacteria (p. 160) where $n = n_0 e^{kt}$.

Here the power kt is positive since the numbers are *increasing* with time.)

The *half-life period* for the decay process is the time after which the concentration of decomposing substance has decreased to half its original concentration, i.e. $n = \frac{n_0}{2}$. Hence the half-life period $t_{\frac{1}{2}}$ is given the expression

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} = \frac{2{\cdot}303 \log 2}{k} = \frac{0{\cdot}693}{k} \quad . \quad . \quad (5.6)$$

Compare this with the mean generation time (m.g.t.) of a bacterial culture (p. 160). The m.g.t. $= \frac{0{\cdot}693}{k}$, where k is the growth constant. The half-life period (or m.g.t.) can be obtained graphically by plotting the logarithm of the number of radioactive atoms (or number of bacteria) against the time and reading off the time interval corresponding to a change of 0·301 (i.e. log 2) in the ordinate. This is shown in Fig. 5.1.

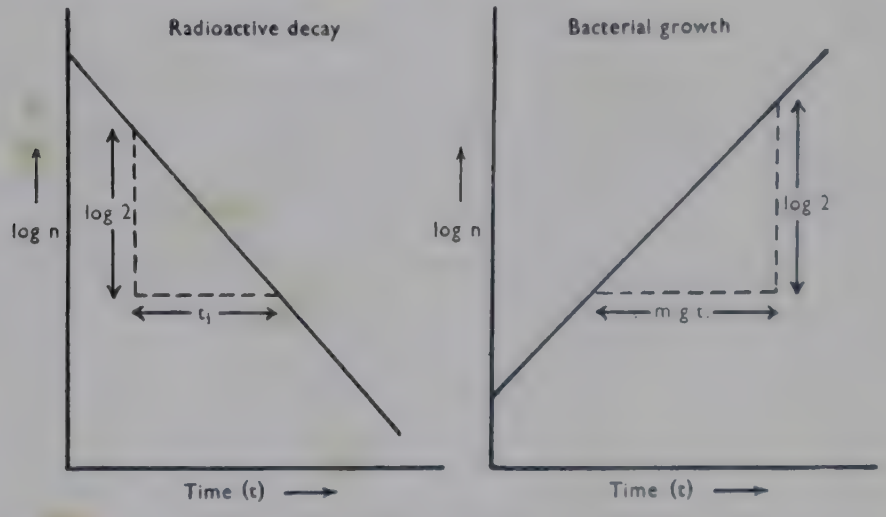

FIG. 5.1

Graphical determination of half-life period in a radioactive decay process and the mean generation time in bacterial growth.

Example 5.1.—A sample of radioactive iodine ^{130}I, giving 316 counts per minute in a Geiger-Müller tube, was assayed at intervals of time. From the following data obtained, determine the half-life period of this isotope.

Time (hours)	5	10	15	18·5	25
Counts/min.	240	182	138	115	79

This problem may be solved either by calculation or graphically. Firstly, by calculation, we have the expression

$$2{\cdot}303 \log \frac{n_0}{n} = kt \quad . \quad . \quad . \quad \text{(equation 5.5)}$$

Here we can substitute for n_0 and n the given values for the counts and thus determine the decay constant k. At zero time $n_0 = 316$ and after 5 hours $n = 240$. Hence

$$k = \frac{2{\cdot}303}{5} \log \frac{316}{240} = \frac{2{\cdot}303 \times 0{\cdot}1195}{5}$$

Furthermore, the half life $t_{\frac{1}{2}}$ is given by equation 5.6

$$t_{\frac{1}{2}} = \frac{0{\cdot}693}{k} = \frac{0{\cdot}693 \times 5}{2{\cdot}303 \times 0{\cdot}1195} = 12{\cdot}6 \text{ hours}$$

Secondly, the graphical solution demands that the logarithm of the radioactive count be plotted against the time.

Time (hours)	0	5	10	15	18·5	25
Counts/min.	316	240	182	138	115	79
Log (Counts/min.)	2·4997	2·3802	2·2601	2·1399	2·0607	1·8976

From the resulting graph, the time interval corresponding to a decrease of log 2 (0·3) is read off and this gives the half-life period, which is 12·5 hours by this method. See Fig. 5.2.

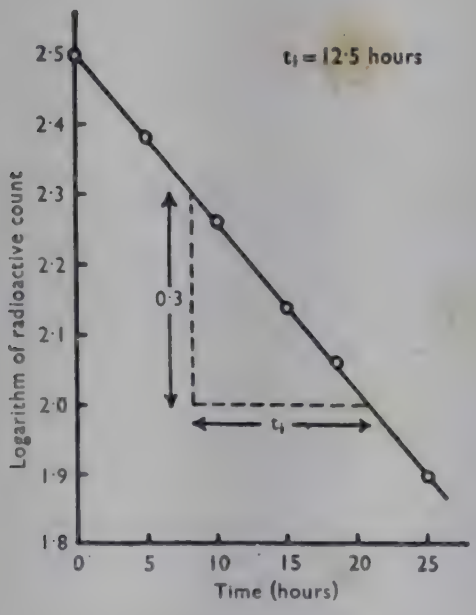

FIG. 5.2.

Second order reactions occur at rates dependent on two concentration terms. Thus, reactions of the type

$$A + B \rightarrow X, Y, \text{ etc.}$$

are of second order and, in fact, this example illustrates the *simplest* type of second order reaction (which is also bimolecular). If the initial concentrations of A and B are (A) = a and (B) = b and at some later time t an amount x has reacted, then the

concentrations of A and B at time t are $(a - x)$ and $(b - x)$. The rate of reaction is thus

$$\frac{dx}{dt} = k(a - x)(b - x) \quad . \quad . \quad . \quad (5.7)$$

Integrating and using the information that $x = 0$ at $t = 0$

$$\ln \frac{a(b - x)}{b(a - x)} = (b - a)kt \quad . \quad . \quad (5.8)$$

or

$$t = \frac{2{\cdot}303}{k(a - b)} \log \frac{b(a - x)}{a(b - x)} \quad . \quad . \quad (5.9)$$

For the special case where $a = b$, or where A and B are both the same substance, the expression becomes

$$\frac{dx}{dt} = k(a - x)^2 \quad . \quad . \quad . \quad (5.10)$$

and

$$\frac{x}{a(a - x)} = kt$$

In more general terms, if C_0 is the initial concentration and C is the concentration at time t, then

$$\frac{1}{C} = \frac{1}{C_0} + kt.$$

Thus, for a second order reaction a plot of $\frac{1}{C}$ against t will be linear.

It will be noted that the rate constant k for a second order reaction is not independent of the units in which the concentrations are expressed but has the dimensions $L^3M^{-1}t^{-1}$. It is usually expressed in litres per mole per second.

Higher Orders of Reaction.—Reactions of third or higher order can also occur, as, for example, in the denaturation of certain proteins. They can be treated in an analogous manner to first and second order reactions as already discussed, but the expressions obtained are naturally more complex.

A method of wide applicability for the study of reaction

velocities is the comparison of reciprocals of the times required to effect a given change. Provided that the initial concentration of reactant is kept constant, then irrespective of the order of the reaction

$$k = \frac{C}{t} \quad . \quad . \quad . \quad . \quad (5.11)$$

where C is a term involving the concentration changes and, by definition, is kept constant. Accordingly under different experimental conditions

$$\frac{k_1}{k_2} = \frac{1}{t_1} \Big/ \frac{1}{t_2} = \frac{t_2}{t_1} \quad . \quad . \quad . \quad (5.12)$$

where k_1 and k_2 are the rate constants under two different conditions and t_1 and t_2 are the times required to effect the same amount of chemical change. In this method no knowledge of the order of the reaction is required, but it is assumed that the order does not change from one set of conditions to the other.

Example 5.2.—The hydrolysis of sucrose to a mixture of glucose and fructose (termed the 'inversion' of sucrose) may be followed by means of a polarimeter. As sucrose is dextro-rotatory and the resulting mixture of glucose and fructose is laevo-rotatory, measurement of the angle of rotation of plane polarised light at various time intervals enables the reaction to be followed. The data given below were obtained by Lewis for the inversion of sucrose in 0·9 N HCl at 25°.

Time (min.)	0·0	7·18	18·00	27·05
Rotation (°)	+24·09	+21·405	+17·735	+15·00
Time (min.)	36·80	56·07	101·70	∞
Rotation (°)	+12·40	+7·80	+0·30	−10·74

Determine the order of the reaction and obtain a value for the velocity constant.

The rotation at zero time, $\alpha_0 = 24{\cdot}09°$, corresponds to pure sucrose and that at infinite time, $\alpha_\infty = -10{\cdot}74°$, corresponds to the complete hydrolysis to glucose and fructose. Consequently the initial concentration of sucrose is proportional to $\alpha_0 - \alpha_\infty = +34{\cdot}83°$ and the concentration of sucrose at any time t, when the rotation is α, is proportional to $\alpha - \alpha_\infty$. The reaction involved is:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow C_6H_{12}O_6 + C_6H_{12}O_6$$

and we might expect, therefore, the reaction either to be of first or of second order. This can be decided by testing the experimental values in the respective equations for these orders or, alternatively, by plotting (*a*) log C against t for first order and (*b*) $\frac{1}{C}$ against t for second order to discover which gives a linear plot.

In either case the velocity constant may be obtained from the slope of the line. Here we shall illustrate this latter method.

Time (min.)	*Rotation* (α)	*Sucrose concn. C* ($\alpha - \alpha_\infty$)	*log C*	$\frac{1}{C}$
0	+24·09 (α_0)	+34·83	1·5420	0·0287
7·18	21·405	32·145	1·5072	0·0311
18·00	17·735	28·475	1·4555	0·0350
27·05	15·00	25·74	1·4106	0·0389
36·80	12·40	23·14	1·3643	0·0432
56·07	7·80	18·54	1·2681	0·0539
101·70	0·30	11·04	1·0429	0·0981
∞	−10·74 (α_∞)	—	—	—

From these figures the graphs are drawn (Fig. 5.3) and it will be seen that the reaction fits the first order equation. From the slope of the line the velocity constant is obtained and has a value of 0·01137 min^{-1}.

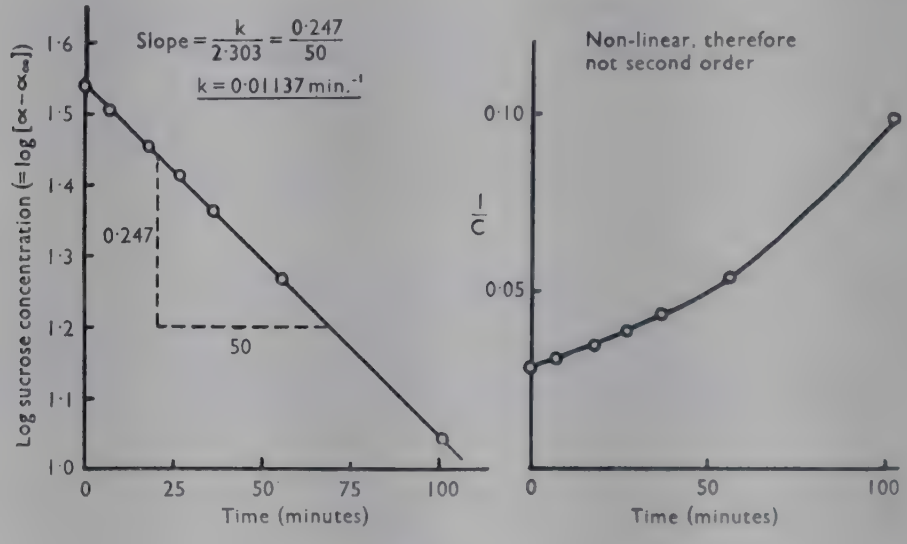

FIG. 5.3

Effect of Temperature on Reaction Rates.

The velocity of a chemical reaction is generally increased by an increase in temperature. The *temperature coefficient* of a reaction, commonly expressed as the Q_{10} value, is the ratio of the velocity at temperature $t + 10°$ to that at temperature $t°$. Q_{10} usually has a value in the region of 2. Enzymic reactions differ from most chemical ones because the temperature increment of velocity usually breaks down between 45 and 50° due to the heat destruction or denaturation of the enzyme protein. Consequently the Q_{10} of an enzyme for the range 40-50° will be generally much less than 2.

Arrhenius, in 1899, proposed the following relationship between

reaction velocity and temperature (compare this with the van't Hoff equation, p. 67).

$$\frac{d \ln k}{dt} = \frac{E}{RT^2} \quad . \quad . \quad . \quad (5.13)$$

where k is the rate constant, R the gas constant (1·987 calories per degree per mole), T the absolute temperature and E is a constant known as the *activation energy*. Integration of the expression between limits T_1 and T_2 (assuming E to be constant over this range) corresponding to rate constants k_1 and k_2 gives

$$\ln \frac{k_2}{k_1} = -\frac{E}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) = \frac{E}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad . \quad (5.14)$$

The value of the activation energy may be obtained from the plot of log k against $1/T$. The slope of the line is $\frac{-E}{2{\cdot}303R}$ from which E can be evaluated. The determination of the energy of activation of enzymic reactions is usually carried out in this way by plotting the logarithm of the reaction velocity against $\frac{1}{T}$ and assuming that the velocity is directly proportional to the rate constant. The Arrhenius equation sometimes applies to complex biological systems taken as a whole, for instance bacterial growth, but the significance of such observations is doubtful and should not be held to imply relation to rate-determining or 'master' reactions in growth (see Monod, 1949).

The Collision Theory of Reaction Rates.

On the collision theory of chemical reactions only a certain proportion of the molecules which collide with one another react. Not every collision results in reaction, and only those molecules possessing sufficient energy will do so. The activation energy E is the 'extra' energy which is required for reaction to occur. Under these conditions the rate of reaction is

$$k = Ze^{-E/RT} \quad . \quad . \quad . \quad (5.15)$$

where k is the number of molecules reacting per second per unit volume, Z is the total number of molecules colliding per second per unit volume and $e^{-E/RT}$ is a measure of the fraction of the

molecules having the necessary energy (or more) to react. The above expression holds fairly well for some reactions but for others it does not, the number of effective collisions (i.e. those resulting in reaction) being other than would be expected from equation 5.15. To allow for this discrepancy an extra term was introduced into the equation, the so-called steric factor, P, which may have any value from unity to zero. This factor is a measure of the probability of the energy that a molecule acquires being distributed in a manner favourable to reaction. Equation 5.15 now becomes

$$k = PZe^{-E/RT} \quad . \quad . \quad . \quad (5.16)$$

In the case of protein denaturation, however, P may turn out to have an astronomical value. For example, in the denaturation of crystalline egg albumin, which has an activation energy of 140,000 calories per mole, P must be of the order of 10^{72}.

The Theory of Absolute Reaction Rates.

An alternative approach to the collision treatment is the theory of absolute reaction rates developed by Eyring and his collabor-

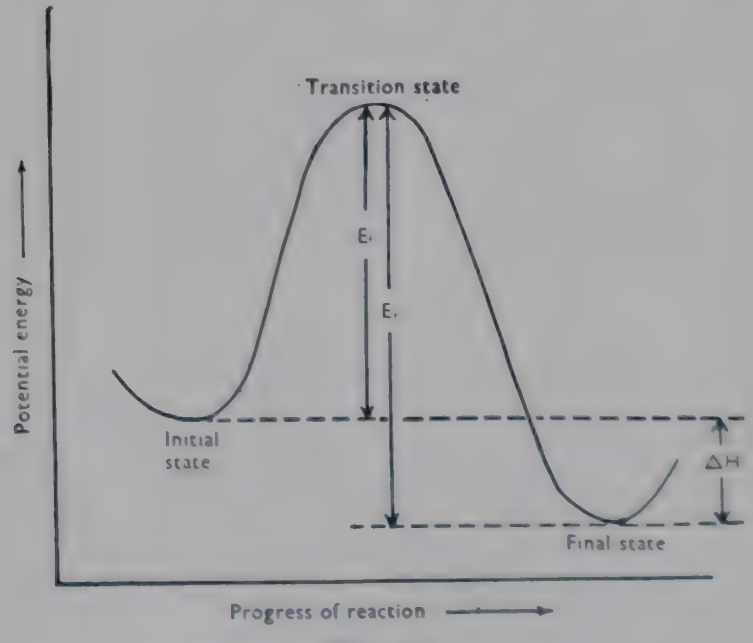

FIG. 5.4

Transition state theory picture of energy of activation.

ators. This treatment is usually known as the *transition state theory*. Here again activation of the reacting molecules is postulated. It is considered that before molecules react they must pass

through a configuration known as the transition state or activated complex which has an energy content greater than either the initial or final states and which are thus separated by an energy barrier. The height of this barrier is the energy of activation and is illustrated in Fig. 5.4. E_f is the activation energy of the forward reaction, i.e. initial to final state. E_r is the activation energy of

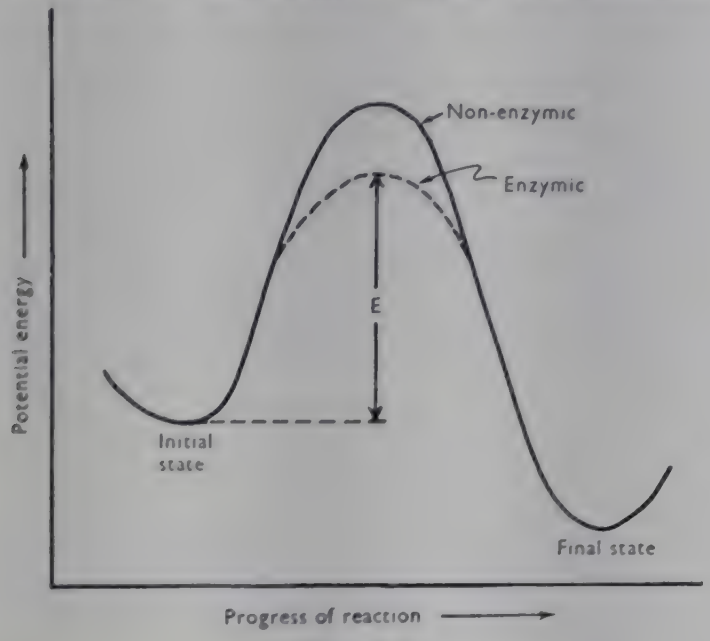

FIG. 5.5

Reduction of energy of activation effected by an enzyme.

the reverse reaction (final to initial) and is greater in value so that the reverse reaction will be much more difficult to accomplish. ΔH is the change in heat content for the reaction. In enzymic reactions the function of the enzyme is to facilitate the reaction by decreasing the energy of activation necessary for the reaction to occur (Fig. 5.5).

Enzymic reactions have, as a consequence, lower temperature coefficients than corresponding non-enzymic ones.

The formation of the transition state can be treated in exactly the same manner as ordinary equilibria, but the symbol ‡ is used to denote functions associated with it. Thus, the equilibrium constant for the reaction resulting in formation of the transition state, $K^{\ddagger}$, is related in the following way to the free energy change

$$\Delta G^{\circ\ddagger} = -RT \ln K^{\ddagger}$$
$$= \Delta H^{\circ\ddagger} - T\Delta S^{\circ\ddagger} \quad . \quad . \quad (5.17)$$

Consider the reaction:

$$A + B \rightarrow \text{Transition State} \rightarrow \text{Products.}$$

The rate of reaction is equal to the concentration of the activated complex at the top of the energy barrier multiplied by the frequency of crossing the barrier. A wave-mechanical approach, outside the scope of this book, is made to this problem; suffice to say that the rate of reaction is equal to $\frac{c^{\ddagger}kT}{h}$, where k is the Boltzmann constant ($1{\cdot}37 \times 10^{-16}$ erg per degree), h is Planck's constant ($6{\cdot}62 \times 10^{-27}$ erg-second), T is the absolute temperature and $c^{\ddagger}$ the concentration of the transition state. The effective rate of crossing the energy barrier is $\frac{kT}{h}$, which is a universal frequency dependent only upon the temperature and independent of the nature of the reactants and the type of the reaction. Now the equilibrium constant, $K^{\ddagger}$, for the formation of the transition state is equal to

$$K^{\ddagger} = \frac{c^{\ddagger}}{c_A c_B} \quad . \quad . \quad . \quad . \quad (5.18)$$

where c_A and c_B are the concentrations of reactants A and B. If the specific reaction rate, i.e. rate constant, is k_r, then the rate of reaction is given by

$$\text{Reaction rate} = k_r c_A c_B$$

Therefore

$$\frac{c^{\ddagger}kT}{h} = k_r c_A c_B$$

and

$$k_r = \frac{kT}{h} \cdot \frac{c^{\ddagger}}{c_A c_B} = \frac{kT}{h} K^{\ddagger} \quad . \quad . \quad (5.19)$$

Substituting this value of $K^{\ddagger}$ in the expression relating it to the standard free energy change we have

$$\Delta G^{\circ\ddagger} = -RT \ln \frac{k_r h}{kT} = \Delta H^{\circ\ddagger} - T\Delta S^{\circ\ddagger} \quad . \quad (5.20)$$

It can be shown that $\Delta H^{\circ\ddagger}$ is related to the energy of activation in the following manner

$$\Delta H^{\circ\ddagger} = E - RT \quad . \quad . \quad . \quad (5.21)$$

Thus the difference between the observed heat of reaction and the activation energy is dependent upon the temperature. At physiological temperatures the difference between the heat change

in the activation process and the activation energy is not great and they may be assumed to be roughly equal.

The transition state theory may be compared now with the collision theory of activation energy. Equation 5.20 may be written in exponential form as

$$k_r = \frac{kT}{h} e^{-\Delta G^{\circ\ddagger}/RT} = \frac{kT}{h} e^{\Delta S^{\circ\ddagger}/R} e^{-\Delta H^{\circ\ddagger}/RT} \quad . \quad (5.22)$$

This shows clearly that the specific reaction rate is determined by the *free energy of activation* at a given temperature and emphasizes the fact that the energy or heat of activation is not the only factor to be considered; what chances exist that the activation energy will be properly used has also to be known.

At physiological temperatures we may equate $\Delta H^{\circ\ddagger}$ and E so that

$$k_r = \frac{kT}{h} e^{\Delta S^{\circ\ddagger}/R} e^{-E/RT} \quad . \quad . \quad (5.23)$$

Comparing this with equation 5.16, we have

$$\frac{kT}{h} e^{\Delta S^{\circ\ddagger}/R} = PZ \quad . \quad . \quad . \quad (5.24)$$

and since $\frac{kT}{h}$ and Z have the dimension of frequency and $e^{\Delta S^{\circ\ddagger}/R}$ and P are dimensionless it follows that

$$\frac{kT}{h} = Z \quad . \quad . \quad . \quad (5.25)$$

and

$$e^{\Delta S^{\circ\ddagger}/R} = P \quad . \quad . \quad . \quad . \quad (5.26)$$

Thus the empirically introduced steric factor P is related to the entropy change involved in the activation reaction. Herein lies the clue to the very high values of P encountered in protein denaturation. The process of denaturation involves very large increases in entropy both for the activation and the overall step, and the steric factor is, therefore, also very large. The values of P may be roughly correlated with the entropy changes as follows:

If $P = 1$ $\Delta S^{\circ\ddagger} = 0$,

$P < 1$ $\Delta S^{\circ\ddagger}$ is negative and the reaction is 'slow',

$P > 1$ $\Delta S^{\circ\ddagger}$ is large and positive.

One final point remains to be made. As we shall see in the next chapter, the kinetics of enzyme action can be satisfactorily explained on the basis of the formation of an enzyme-substrate complex. It would be tempting to assume that this complex is identical with the transition state, but according to experiments carried out on the peptic hydrolysis of egg albumin by Bull and Currie (1949), this is not the case. It would seem that the enzyme-

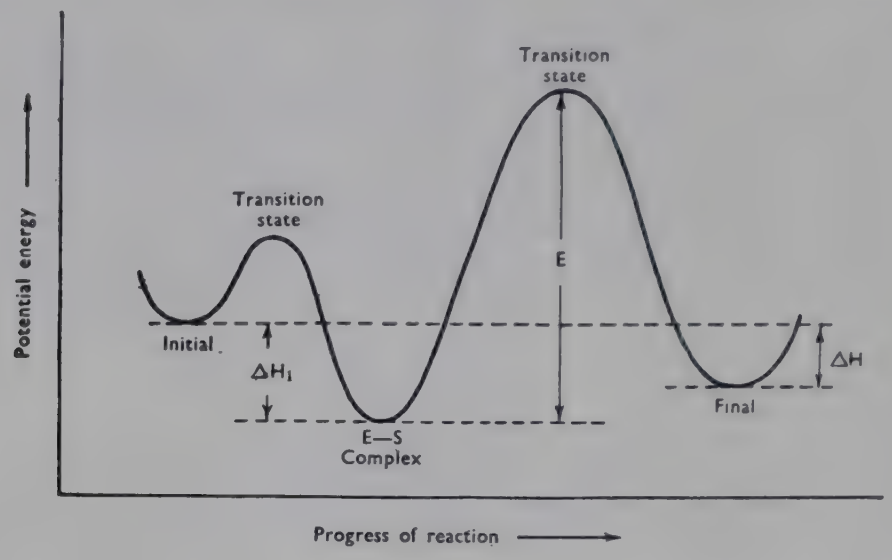

FIG. 5.6

Relationship of the enzyme-substrate complex of Michaelis-Menten treatment to the transition state theory of reactions. (After GIBSON, 1953.)

substrate complex must pass into an activated state before it can decompose into the final products of the reaction. This situation is depicted in Fig. 5.6. The enzyme-substrate complex is formed, presumably after passing through a transition state, and the change in heat content corresponding to this step is ΔH_1. The activation energy E is required to convert the enzyme-substrate complex to the products of the reaction, passing through a second transition state. ΔH is the change in heat content for the overall reaction.

Example 5.3.—Write down an expression for the effect of change in temperature on the equilibrium constant of a reversible reaction.

Trypsin is reversibly denatured by heating. At 44° C. the equilibrium constant for the transformation of native to denatured trypsin is 1·00 and at 50° C. it is 7·20. Calculate the changes occurring in heat content and entropy at 44° C.

(Leeds Honours Course Finals, 1951.)

The required expression is $\dfrac{d \ln K}{dT} = \dfrac{\Delta H}{RT^2}$.

To calculate the change in heat content, this is required in its integrated form

$$\ln \frac{K_2}{K_1} = \frac{\triangle H}{R}\left(\frac{T_2 - T_1}{T_1 T_2}\right).$$

Substituting the values of K at 317° and 323° absolute (44° and 50° C.) and R (1·987 calories per degree per mole)

$$\triangle H = \frac{2{\cdot}303 \times 1{\cdot}987 \times \log 7{\cdot}2 \times 323 \times 317}{(323 - 317)}$$

$$= \frac{2{\cdot}303 \times 1{\cdot}987 \times 0{\cdot}8573 \times 323 \times 317}{6}$$

$$= \underline{67{,}150 \text{ calories.}}$$

Taking the same activity coefficients for native and denatured protein, the relation between equilibrium constant and free energy change becomes $\triangle G = -RT \ln K$ and $\triangle G$ is related to the change in heat content and entropy by the expression $\triangle G = \triangle H - T\triangle S$. But at 317° absolute $K = 1$, therefore $\ln K = 0$ and $\triangle G = 0$.

Hence $\triangle H = T\triangle S$.

Therefore $\triangle S = \frac{67150}{317} = \underline{211{\cdot}8 \text{ entropy units.}}$

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PROBLEMS

5.1. A sample of radioactive iron, ^{59}Fe, was assayed in a Geiger-Müller tube at intervals of time and the following data obtained.

Time (days)	*Radioactive counts per minute*
0	3981
2	3864
6	3648
10	3437
14	3238
20	2965

Determine the half-life period and evaluate the decay constant.

5.2. The kinetics of the aerobic oxidation of enzymatically reduced diphosphopyridine nucleotide (DPNH) have been investigated at pH 7·38 and 30° C. The reaction rate was followed spectrophotometrically by measuring the

decrease in optical density at 340 mμ. over a period of 30 minutes. The catalysed reaction may be represented as

$$DPNH + H^+ + \text{riboflavin} \rightarrow DPN^+ + \text{riboflavin } H_2 \text{ (leuco-riboflavin)}$$

Time (minutes)	*Optical density at 340 mμ.*
1	0·347
2	0·339
5	0·327
9	0·302
16·5	0·275
23	0·254
27	0·239
30	0·229

Determine the rate constant and the order of the reaction.

(After SINGER & KEARNEY (1950), *J. biol. Chem.*, **183**, 409.)

5.3. The effect of substrate concentration on the initial rate of the fumarase reaction, which catalyses

$$\text{fumarate}'' + H_2O \rightleftharpoons \text{malate}''$$

has been investigated in both directions at pH 7·29. The experimental figures obtained are recorded below.

Substrate concentration	*Initial rate*, mole/sec. × 10^7	
M	*Fumarate*	*Malate*
0·40	5·0	2·8
0·20	6·0	3·2
0·10	5·9	3·2
0·05	5·6	4·0
0·025	5·4	2·5
0·0125	6·0	3·0

Determine the order of the reaction in the initial stages. What explanation can you offer for your findings?

(After SCOTT & POWELL (1948), *J. Amer. chem. Soc.*, **70**, 1104.)

5.4. The relationship between the initial rate of reaction and temperature for the fumarase system (see Problem 5.3) has been investigated in both directions and the following data were obtained at pH 7·29.

Initial rates mole/sec. × 10^7		*Temperature*
Fumarate	*Malate*	° C.
15·85	9·550	50
14·13	5·623	45
10·00	4·467	40
8·913	3·548	35
6·310	2·818	30
5·012	1·778	25
3·981	1·259	20
3·162	0·8511	15
2·818	0·6310	10

Calculate the apparent activation energies when the substrate is (*a*) fumarate and (*b*) malate.

(After SCOTT & POWELL (1948), *J. Amer. chem. Soc.*, **70**, 1104.)

5.5 In an experiment to obtain an estimate of the heat of activation of the breakdown of tryptophan to indole by the tryptophanase enzyme of *Escherichia coli* indole production was measured at various intervals of time at four different temperatures. Results obtained are recorded below.

Time (min.)	*μg. Indole produced at temperature* (°C.)			
	0	14·5	27	37
5			7·0	25·0
10		3·7	15·3	27·0
15			24·5	28·6
20		6·4		30·8
25			25·4	
30	2·1	12·8	26·0	
40		16·0		
50		17·0		
60	4·5	17·0		

Use these data to obtain a value for the heat of activation of the tryptophanase reaction.

(After HAPPOLD & MORRISON, quoted by HAPPOLD (1950), *Advances in Enzymol.*, **10**, 66.)

5.6. Heat denaturation of the proteolytic enzyme trypsin is a reversible process and may be followed by measuring the loss of enzymic activity which occurs when the active native trypsin is denatured to the inactive form. The reaction may be represented

$$\underset{\text{active}}{\text{Trypsin (native)}} \rightleftharpoons \underset{\text{inactive}}{\text{Trypsin (denatured)}}$$

The effect of temperature on the equilibrium of the reaction was investigated by Anson & Mirsky and some of their results are given below.

Temperature °C.	*Percentage denaturation*
42	32·8
43	39·2
44	50·0
45	57·4
48	80·4
50	87·8

Use this information to determine the heat of reaction of the denaturation process and also obtain a value for the change in entropy which occurs. Comment on the values obtained for ΔH and ΔS.

(After ANSON & MIRSKY (1933-34), *J. gen. Physiol.*, **17**, 393. Glasgow Honours Course Finals, 1954.)

5.7. The energy of activation of the hydrolysis of sodium β-glycerophosphate by bone phosphatase has been determined by Bodansky using both cat and human bone phosphatases.

The phosphatase preparations were incubated at various temperatures with sodium β-glycerophosphate at the optimal pH (9·0 – 9·2) in a suitable buffer solution in the presence of glycine and magnesium ions. The conditions were such that the reaction velocity was directly proportional to the concentration of the enzyme. The reaction velocity was determined by measuring the amount of phosphorus liberated as inorganic phosphate per ml. of hydrolysis

mixture during the portion of the reaction which is of zero order. Results obtained are given in the following table:

Temperature	*Phosphorus liberated as phosphate per ml. hydrolysis mixture per minute.*	*Number of experiments performed*
	Action of cat bone phosphatase	
°C.	mg.	
12·00	0·000425	2
17·30	0·000625	2
20·90	0·000819	3
27·05	0·00111	1
27·10	0·00117	1
31·70	0·00147	1
32·00	0·00142	1
37·50	0·00195	3
42·40	0·00243	2
	Action of human bone phosphatase	
20·00	0·000664	2
25·00	0·000863	2
30·00	0·00103	2
35·00	0·00150	2
40·00	0·00187	2

Use these data to obtain a value for the energy of activation of the hydrolytic reaction.

(After BODANSKY (1939), *J. biol. Chem.*, **129**, 197.)

5.8. The Michaelis constant of the citric dehydrogenase of cucumber seeds has been determined at two different temperatures. Dann obtained the following values in five determinations at each temperature:

25° C. 142 281 137 130 137 millimolar
35° C. 65 71 70 115 78 millimolar

Calculate the heat of formation of the enzyme-substrate complex from this information.

(After DANN (1931), *Biochem. J.*, **25**, 177.)

5.9. The tropine esterase activity of rabbit serum has been investigated by measuring the acid produced when atropine sulphate is hydrolysed by the enzyme. Continuous titration in buffer-free medium with a glass electrode enabled the reaction to be expressed in terms of ml. 0·02 N NaOH required to neutralize the liberated acid. The following data show tropine esterase activity as a function of temperature; the figures refer to 30 minutes' action by 2·5 per cent. rabbit serum on 0·25 per cent. atropine sulphate, which provides excess substrate, at pH 8·4.

ml. 0·02 N NaOH *per* 30 *min.*		*Temperature* °C.
Enzyme	*Control*	
0·09	0·05	20
0·14	0·06	28
0·175	0·07	32

ml. 0·02 N NaOH *per* 30 *min.*		*Temperature* °C.
Enzyme	*Control*	
0·24	0·08	36
0·255	0·09	38
0·24	0·10	40
0·19	0·125	44·5

Calculate the activation energy of the tropine esterase reaction.

(After GLICK (1940), *J. biol. Chem.*, **134**, 617.)

5.10. Kiese determined the effect of temperature on the Michaelis constant of carbonic anhydrase which catalyses the reaction

$$H_2O + CO_2 \rightleftharpoons H_2CO_3.$$

He obtained the following values in 0·04 M phosphate buffer at pH 7·4

Temperature °C.	$K_s \times 10^3$
1·0	1·2
5·0	2·3
8·0	2·9
12·5	5·2

Use this information to determine the heat of formation of the enzyme-substrate complex.

(After KIESE (1941), *Biochem. Z.*, **307**, 400.)

5.11. The reaction velocity of the citric dehydrogenase of cucumber seeds has been measured by the Thunberg tube technique. The citrate concentration used was large enough to ensure complete saturation of the enzyme and measurements were made at 25° and 35° with the same enzyme preparation. The ratio of the rate constants k_{35}/k_{25} in three different experiments gave values of 1·61, 1·70 and 1·64. Determine the heat of activation of the citric dehydrogenase-citric acid complex.

(After DANN (1931), *Biochem. J.*, **25**, 177.)

5.12. The action of 0·2 per cent. phenol in killing *B. typhosus* has been investigated by means of viable counts of the organisms at various intervals of time. The following table records the mean viable count of triplicate plates at time intervals following the addition of phenol:

Time (*min.*)	*Mean number of micro-organisms surviving*
0	20400
2	18000
4	11600
6	8000
8	6400
10	5200
15	2800
20	1500
25	750
30	400
35	250
40	120
45	64

Determine whether the killing process displays well defined kinetics, and, if so, its characteristics.

(After LEE & GILBERT (1917-18), *J. phys. Chem.*, **22**, 348.)

5.13. The following data were obtained in studies on the rates of spontaneous (non-enzymatic) decarboxylation of oxaloacetic and oxalosuccinic acids.

Each Warburg manometer contained 1·5 ml. of 0·3 M acetate buffer, pH 5·1, made up with keto acid and water to a volume of 2·5 ml. Oxaloacetic acid (19 μmoles) and oxalosuccinic acid (16 μmoles) were tipped into the main compartment after temperature equilibration at 25°. The gas phase was air.

Oxaloacetic acid		*Oxalosuccinic acid*	
Time (min.)	*CO_2 evolved (μl.)*	*Time (min.)*	*CO_2 evolved (μl.)*
15	16	5	47
30	40	10	89
45	60	15	122
60	74	20	150
120	142	25	170
		30	189

Determine the order of the reaction and the velocity constant for the spontaneous decarboxylation of each keto acid.

(After OCHOA (1948), *J. biol. Chem.*, **174**, 115.)

5.14. Myosin catalyses the reaction

$$ATP \rightarrow ADP + \text{inorganic phosphate.}$$

The kinetics of the thermal deactivation of myosin have been studied by incubation of identical solutions of myosin at three different temperatures, followed by addition of ATP solution (0·002 M) and determination of the rates of production of inorganic phosphate. These rates, called activities, are recorded below for experiments conducted at pH 7·0 with an enzyme concentration of 0·09 g./l.

Temperature °C.	*Time sec.*	*Activity μmole phosphate/sec.*
35·2	90	0·873
	320	0·776
	776	0·600
	1400	0·415
	2182	0·272
30·2	70	0·685
	1500	0·600
	2610	0·536
25·0	1000	0·453
	74000	0·175

Determine the heat of activation of the thermal deactivation process.

(After OUELLET, LAIDLER & MORALES (1952), *Arch. Biochem. Biophys.*, **39**, 37.)

5.15. A study of the non-enzymic hydrolysis of adenosine triphosphate (ATP) is of interest for comparison with the enzymic reaction apparently occurring during the functioning of a muscle. The following data on the catalysed hydrolysis of ATP are taken from Friess.

The reaction

$$ATP + H_2O \xrightarrow{H^+} ADP + H_3PO_4$$

was studied at pH 1·33 in the presence of 0·300 M sodium chloride. At 50·24° C. the following values were obtained :

ATP (moles/litre)	*Time (sec.)*
·0198	0
·0188	3000
·0183	5600
·0166	12300
·0161	15000
·0156	17000
·0150	19200
·0145	21400

Does the reaction obey first order kinetics with respect to ATP ? What is the rate constant ? Is the reaction monomolecular or bimolecular ? How might you test this experimentally ? With $(ATP)_0 = 0{\cdot}0198$ M, pH = 1·33, NaCl = 0·300 M the following rate constants were calculated :

Temp. °C.	k_1 *(sec.*$^{-1}$*)*
39·94	$4{\cdot}67 \times 10^{-6}$
43·82	$7{\cdot}22 \times 10^{-6}$
47·06	$10{\cdot}0 \times 10^{-6}$
50·24	$13{\cdot}9 \times 10^{-6}$

Plot these data according to the Arrhenius equation. What is the activation energy ?

Calculate $\triangle G^{\ddagger}$, $\triangle H^{\ddagger}$ and $\triangle S^{\ddagger}$ at the reference temperature of 40·0 °C.

(After FRIESS (1953), *J. Amer. chem. Soc.*, **75**, 323. Harvard Medical Sciences 201 ab.)

5.16. Snyder and Snyder give the following observations for the rate of flashing of fireflies as a function of temperature :

Temperature of air °C.	*Rate of flashings per minute* (k)
28·3	15·0
28·8	15·4
26·0	12·6
22·6	10·0
22·3	9·9
23·2	11·1
24·1	11·5
26·5	12·1
19·4	8·1

Calculate the Arrhenius activation energy, E, for the process.

Taking k at 19·4° as the reference standard, use the calculated value of E to compute the value of k at 26·0°, and compare the computed and observed values of k.

(After SNYDER & SNYDER (1920), *Amer. J. Physiol.*, **51**, 536. Harvard Medical Sciences, 201 ab.)

CHAPTER VI

ENZYME KINETICS

The kinetics of enzyme action were first explained satisfactorily by Michaelis and Menten (1913) with the postulate that the enzyme combines reversibly with its substrate to form an intermediate complex. During recent years Chance and his collaborators have been able to demonstrate the existence of such enzyme-substrate complexes in certain reactions by very delicate spectroscopic techniques. This complex then breaks down to form the product(s) of the reaction and liberates the enzyme for further reaction. Application of the law of mass action to the reversible reaction enables an expression to be derived relating the velocity of reaction with substrate and enzyme concentrations.

Consider an enzyme E reacting with substrate S

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{E{-}S} \xrightarrow{k_3} \mathrm{P} + \mathrm{E},$$

where E–S is the enzyme-substrate complex and P the product(s).

Let s be the concentration of substrate,

e the concentration of total enzyme and

c the concentration of enzyme-substrate complex.

By the law of mass action the velocity of the forward reaction, i.e. formation of E–S, is proportional to the concentration of the reactants

$$v_1 = k_1(e - c)s$$

The velocity of the reverse reaction is

$$v_2 = k_2 c$$

At equilibrium $v_1 = v_2$ and $k_1(e - c)s = k_2 c$

$$\frac{(e - c)s}{c} = \frac{k_2}{k_1} = K_s$$

$$\frac{es}{c} - s = K_s$$

$$c = \frac{es}{K_s + s} \quad . \quad . \quad . \quad (6.1)$$

c is the concentration of the enzyme-substrate complex, the breakdown of which is assumed to govern the rate of formation of the products of the reaction, i.e. the overall velocity of the reaction V. The assumption is made, therefore, that the rate of formation of the enzyme-substrate complex is in excess of its rate of decomposition; in other words, $k_1 > k_2 \gg k_3$ and the decomposition of the complex to the products of the reaction is the rate-determining step.

Hence $V = k_3 c$ and substituting for c in equation 6.1

$$V = \frac{k_3 es}{K_s + s}$$

When V is the maximum rate of reaction, i.e. $V = V_{max.}$, then all the enzyme is in the form of enzyme-substrate complex, the reaction is of zero order with respect to substrate and $V_{max.} = k_3 e$, so that

$$V = \frac{V_{max.} s}{K_s + s} \quad . \quad . \quad . \quad (6.2)$$

Rearranging $$K_s = s\left(\frac{V_{max.}}{V} - 1\right) \quad . \quad . \quad (6.3)$$

and these are the forms of the Michaelis-Menten equation. Note that an equation of exactly similar form is obtained by application of the Langmuir adsorption theory to enzyme reactions. K_s, which under these defined conditions is actually the dissociation constant of the enzyme-substrate complex, is known as the *Michaelis constant* and is a very important value, being a characteristic of each enzyme. It is independent of either enzyme or substrate concentration and may be defined as *the substrate concentration at half maximum velocity of reaction*. This is readily appreciated from equation 6.3 since when $V = \frac{V_{max.}}{2}$, then $K_s = s$. The Michaelis constant is always determined when an enzyme is being characterized. It can be done by plotting the

initial velocity of the reaction versus the substrate concentration and hence determining the maximum velocity. From this the substrate concentration at half maximum velocity can be obtained graphically (Fig. 6.1).

The units in which K_s is measured are, therefore, units of concentration and it is customary to express them as moles per litre.

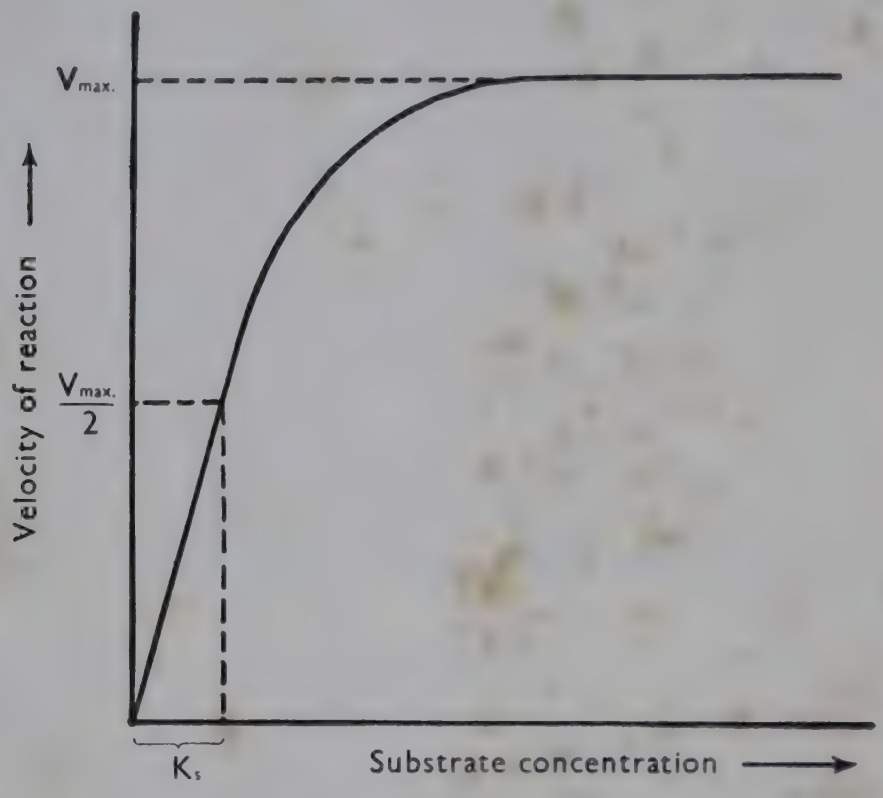


FIG. 6.1
Graphical determination of the Michaelis constant, K_s, of an enzyme.

An alternative and more accurate graphical method was introduced by Lineweaver and Burk (1934), who plotted the reciprocals of velocity and substrate concentrations and thus obtained a straight line (Fig. 6.2). Taking the reciprocal of equation 6.2

$$\frac{1}{V} = \frac{K_s + s}{V_{max.}\, s}$$

$$\frac{1}{V} = \frac{K_s}{V_{max.}\, s} + \frac{1}{V_{max.}} \quad . \quad . \quad . \quad (6.4)$$

This is the equation of a straight line with slope of $\frac{K_s}{V_{max.}}$ and

intercept of $\frac{1}{V_{max.}}$ on the $\frac{1}{V}$ axis. From the graph the values of $V_{max.}$ and K_S can be evaluated.

It should be noted that in deriving the Michaelis-Menten equation certain assumptions are made which for some systems are justified. More complicated treatment, outside the scope of this book, is necessary to cover the other instances. Particular

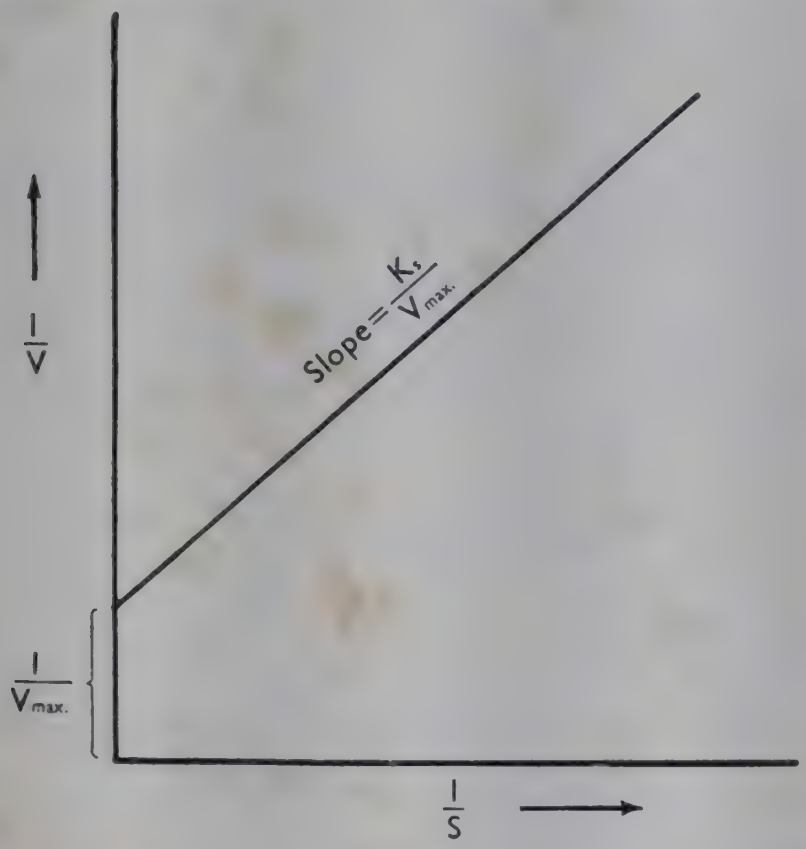


FIG. 6.2

Determination of Michaelis constant by Lineweaver-Burk reciprocal plot.

emphasis must be placed on the fact that K_S can only be equated with the dissociation constant of the enzyme-substrate complex (k_2/k_1) when k_2 is very much greater than k_3. Where this does not apply, $K_S = (k_2 + k_3)/k_1$.

In many cases an enzyme will function only in the presence of a coenzyme which is attached to the enzyme protein. The combination of enzyme with coenzyme can be subjected to exactly the same treatment as enzyme-substrate complex formation and hence the dissociation constant of the enzyme-coenzyme complex may be found.

Example 6.1. The D-serine dehydrase of *Neurospora crassa* has been shown

to require pyridoxal phosphate as coenzyme. The enzyme catalyses the reaction

$$CH_2OH.CHNH_2.COOH \rightarrow CH_3CO.COOH + NH_3.$$

The following figures were obtained in an experiment to determine the pyridoxal phosphate saturation curve of the enzyme.

μMoles pyruvic acid formed in 20 minutes	*Pyridoxal phosphate concentration* ($\times 10^5$ M)
0·150	0·20
0·200	0·40
0·275	0·85
0·315	1·25
0·340	1·70
0·350	2·00
0·360	8·00

Use these data to determine the dissociation constant of the serine dehydrase-pyridoxal phosphate complex. (After YANOFSKY (1952), *J. biol. Chem.*, **198**, 343.)

From the reaction catalysed it will be seen that pyruvic acid formation is a measure of the velocity of the reaction. The required constant may be obtained graphically by plotting either (1) the velocity of reaction V against coenzyme concentration s or (2) $1/V$ against $1/s$, the Lineweaver-Burk method.

The graph for method 1 is shown in Fig. 6.3. From this the value of K obtained is $3{\cdot}2 \times 10^{-6}$ M.

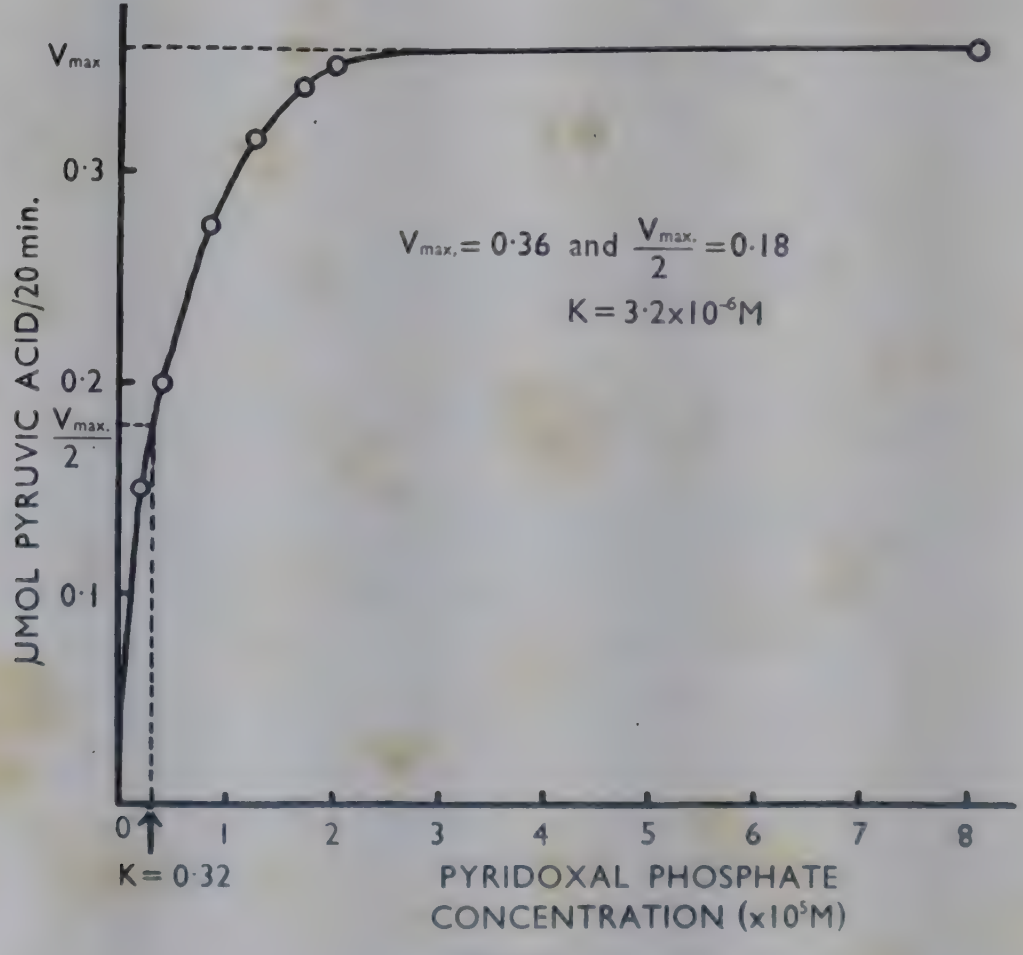


FIG. 6.3

Method 2 requires that the reciprocals of the velocity of reaction and coenzyme concentration be obtained.

Velocity (V) *μmoles pyruvic acid*	$\frac{1}{V}$	*Coenzyme concentration* (s) ($\times 10^5$ M)	$\frac{1}{s}$ ($\times 10^{-5}$)
0·150	6·66	0·20	5·0
0·200	5·00	0·40	2·5
0·275	3·64	0·85	1·17
0·315	3·17	1·25	0·80
0·340	2·94	1·70	0·58
0·350	2·86	2·00	0·50
0·360	2·87	8·00	0·125

These values are plotted in the graph in Fig. 6.4 from which measurements of the slope and intercept enable K to be evaluated; in this case a value of $3{\cdot}5 \times 10^{-6}$ M is obtained. This method gives more reliable results than the former.

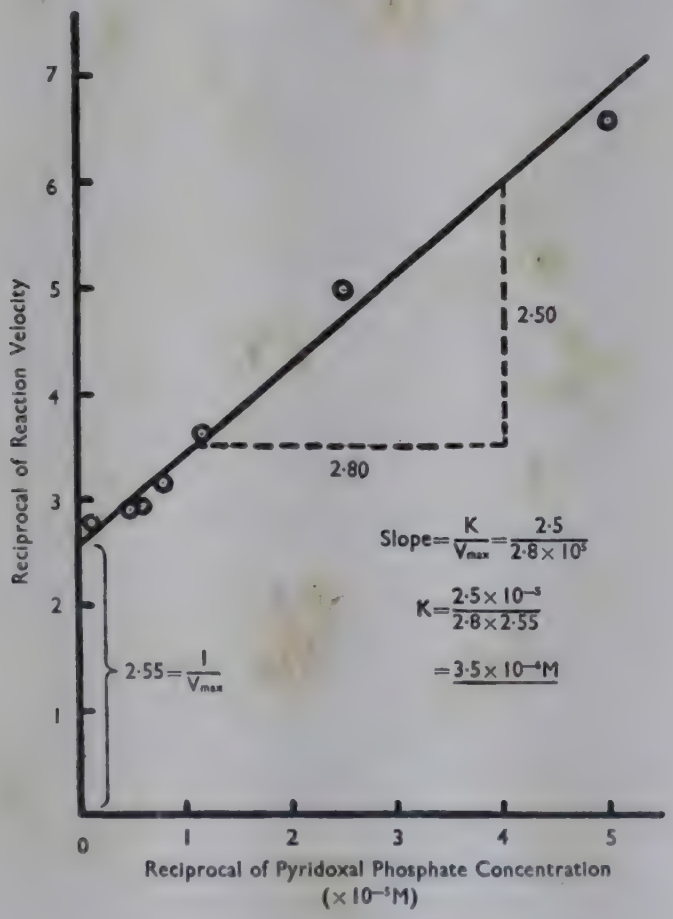


FIG. 6.4

In the above example the concentration of the pyridoxal phosphate was expressed in terms of molarity, thus facilitating the calculation of the required dissociation constant. Had the concentration been given in terms of, say, milligrams per litre, it

would have been necessary to convert this to molarity by dividing the concentration in *grams* per litre by the molecular weight, i.e.

$$\frac{\text{milligrams per litre} \times 10^{-3}}{\text{molecular weight}}.$$

It is possible to obtain the value of K_s by a simpler graphical method which, as Dixon (1953) points out, Lineweaver and Burk

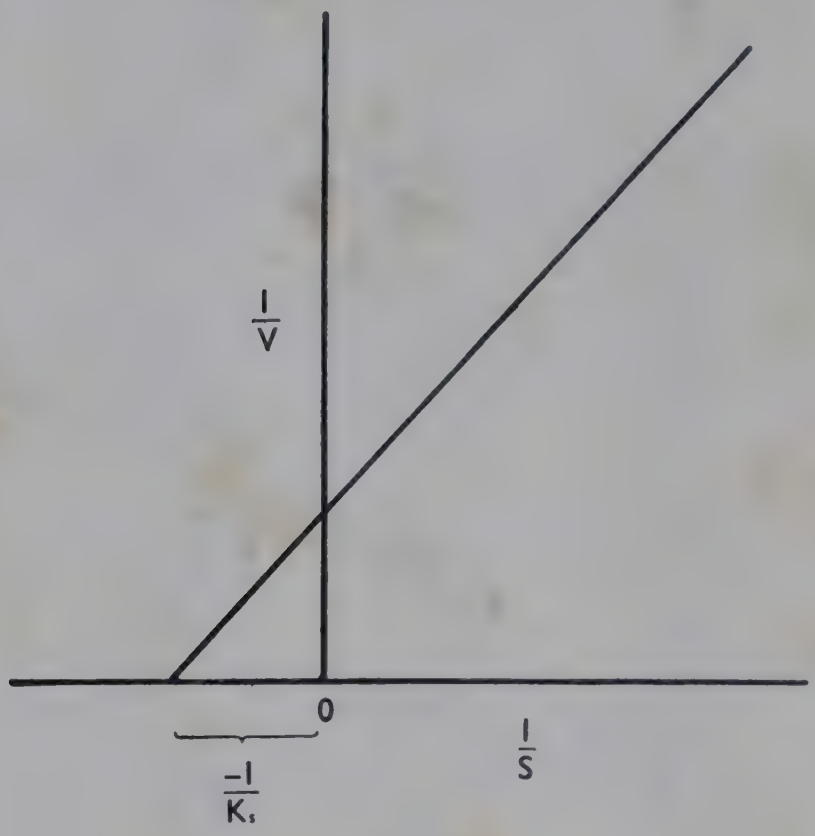


FIG. 6.5
Determination of Michaelis constant.

did not mention. Schwimmer (1950), however, has used this method in published work. As before, $1/V$ is plotted against $1/s$ and if the resulting straight line is produced to the left of the ordinate axis it will cut the abscissa, giving an intercept of $-1/K_s$ (Fig. 6.5). This is evident by putting $1/V = 0$ in equation 6.4, which then becomes $1/s = -1/K_s$.

ENZYME INHIBITION

An understanding of the kinetics of enzyme action is very useful for determining the mode of action of certain inhibitors of enzyme

catalysed reactions. Generally speaking, inhibitors can be divided into two groups—specific and non-specific. Non-specific inhibition implies that the inhibitor exerts its action on the enzyme protein causing denaturation, i.e. the agent will inhibit any enzyme and does not act solely upon the active centre or point of combination with the substrate. A specific inhibitor, on the other hand, exerts its action by combining with the active centre or, perhaps, with the coenzyme, thus preventing enzyme-substrate combination. This type of inhibition depends upon the individual reactive centres of enzymes. Two types of specific inhibition may be encountered—competitive and non-competitive.

Where the specificity is not absolute, the enzyme may combine with a compound which bears a structural similarity to its true substrate (or to its coenzyme), combination with the latter is prevented and enzyme action inhibited, i.e. there is competition between substrate and inhibitor for the enzyme centre. The classical example of competitive inhibition is the inhibition of succinic acid dehydrogenase by malonic acid, the lower homologue of the true substrate. The degree of inhibition is dependent on the relative concentrations of malonic and succinic acids; by increasing the succinic acid concentration relative to that of malonic acid the inhibition is reversed and eventually overcome. The combination between inhibitor and enzyme is readily reversible and the degree of inhibition is dependent upon both substrate and inhibitor concentrations.

Non-competitive inhibition occurs when a specific and reversible inhibition is produced which is dependent upon inhibitor concentration only and which cannot be reversed simply by increasing the substrate concentration. This indicates that the specific combination of inhibitor with enzyme occurs at some point essential for activity but not at the active centre where substrate combination occurs.

It must be emphasized that the distinction between the various types of inhibition by selected agents applies to individual enzymes only. Thus an inhibitor may act competitively with one enzyme, non-competitively with another and non-specifically with a third; each enzyme must be assessed individually.

The difference between competitive and non-competitive inhibitors may be determined experimentally and the method is based upon the following derivations.

Competitive Inhibition (Lineweaver & Burk, 1934).

The reversible reactions occurring are:

$$(a)\ \mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{E{-}S} \xrightarrow{k} \mathrm{E} + \mathrm{P}$$

$$(b)\ \mathrm{E} + \mathrm{I} \rightleftharpoons \mathrm{E{-}I}$$

where I is the inhibitor.

Let e = concentration of enzyme

s = concentration of substrate

i = concentration of inhibitor

c = concentration of enzyme-substrate complex

d = concentration of enzyme-inhibitor complex

K_s = dissociation constant of E—S

K_1 = dissociation constant of E—I.

Applying the law of mass action

$$K_s = \frac{(e - c - d)s}{c}$$

$$K_1 = \frac{(e - c - d)i}{d}$$

Eliminating d,

$$\frac{(e - c)i}{K_1 + i} = d$$

$$K_s c = s\left(e - c - \frac{ei - ci}{K_1 + i}\right)$$

$$= s\left(\frac{eK_1 - cK_1}{K_1 + i}\right)$$

$$c = \frac{esK_1}{K_sK_1 + K_si + K_1s}$$

Now, as before, $V = \dfrac{kesK_1}{K_sK_1 + K_si + K_1s}$

and at enzyme saturation $V_{\text{max.}} = ke$

$$V = \frac{V_{\text{max.}}\, sK_1}{K_sK_1 + K_si + K_1s} \quad . \quad . \quad . \quad (6.5)$$

Taking the reciprocal of both sides

$$\frac{1}{V_i} = \frac{K_s K_1 + K_s i + K_1 s}{V_{max.}\, s K_1}$$

$$\frac{1}{V} = \frac{K_s}{V_{max.} s} + \frac{K_s i}{V_{max.} s K_1} + \frac{1}{V_{max.}}$$

or

$$\frac{1}{V_i} = \frac{K_s}{V_{max.} s}\left(1 + \frac{i}{K_1}\right) + \frac{1}{V_{max.}} \quad . \quad . \quad (6.6)$$

where V_1 is the velocity and $V_{max.}$ the maximum velocity in the presence of the inhibitor.

Comparison of equation 6.6 with equation 6.4 shows that the effect of a competitive inhibitor is to increase by the value $\frac{K_s i}{K_1 V_{max.}}$ the slope of the line obtained when $\frac{1}{V}$ is plotted against $\frac{1}{s}$. Consequently when there is an increase in the slope of the $\frac{1}{V}$ plot, accompanied by no significant change in intercept, competitive inhibition is indicated. The value of K_1 may be obtained from the slope of the plotted line, since the slope is equal to $\frac{K_s}{V_{max.}}\left(1 + \frac{i}{K_1}\right)$. Clearly the values of K_s and i must be known. At equal concentrations of two inhibitors the slope will be greater in the case of the more potent inhibitor, i.e. a higher $\frac{1}{K_1}$ value.

An alternative formulation may be obtained from equations 6.4 and 6.6

$$\frac{V}{V_1} = 1 + \frac{K_s}{K_1}\left(\frac{i}{K_s + s}\right) \quad . \quad . \quad (6.7)$$

In this case, if V/V_1 is plotted against different values of inhibitor concentration i, straight lines are obtained with unit intercept and with slope dependent on s.

Non-competitive Inhibition.

When non-competitive inhibition occurs, the following reactions are involved:

$$E + S \rightleftharpoons E{-}S \rightarrow E + P$$

$E + I \rightleftharpoons E—I$ (inactive with dissociation constant K_1)

$E—S + I \rightleftharpoons E—S—I$ (inactive with dissociation constant $K_{esi} = K_1$).

In a similar manner to that employed for competitive inhibition it can be shown that

$$\frac{1}{V_1} = \left(1 + \frac{i}{K_1}\right)\left[\frac{1}{V_{max.}} + \left(\frac{K_s}{V_{max.}}\right)\frac{1}{s}\right] \quad . \quad (6.8)$$

and

$$\frac{V}{V_1} = 1 + \frac{i}{K_1} \quad . \quad . \quad . \quad . \quad . \quad (6.9)$$

Here, as Wilson (1949) points out, the effect of a non-competitive inhibitor is to increase *both slope and intercept* by the factor $\left(1 + \frac{i}{K_1}\right)$ when $1/V$ is plotted against $1/s$. This affords a ready means of differentiation of competitive and non-competitive inhibitors since in the former case the intercept does not increase.

Equation 6.9 shows that the plot of V/V_1 against i gives a straight line with unit intercept and slope of $1/K_1$, independent of s. With a competitive inhibitor, as already seen, the slope is dependent on s and this affords a second method for distinguishing between the two types of inhibition. Fig. 6.6 illustrates these differences for the plot of $1/V$ against $1/s$ and Fig. 6.7 for V/V_1 against i. Thus the type of inhibition produced by given substances may be decided by graphical means and it is usually possible to determine the dissociation constant of the enzyme-inhibitor complex.

A simpler graphical method of obtaining values for K_1 in the case of competitive and non-competitive inhibitors has been described by Dixon (1953). With a competitive inhibitor, as previously seen, the effect of varying independently both s and i must be determined in order to obtain K_1. However, if $1/V_1$ is plotted against i, keeping s constant, a straight line will be obtained, and if this is done at two different substrate concentrations, s_1 and s_2, the resultant lines will intersect at a point to the left of the ordinate axis, as shown in Fig. 6.8. This point lies at a value of $-K_1$ which can thus be read directly from the graph. This may be proved from equation 6.6 as follows:

$$\frac{1}{V_1} = \frac{K_s}{V_{max.}}\left(1 + \frac{i}{K_1}\right)\frac{1}{s} + \frac{1}{V_{max.}} \quad . \quad (6.6)$$

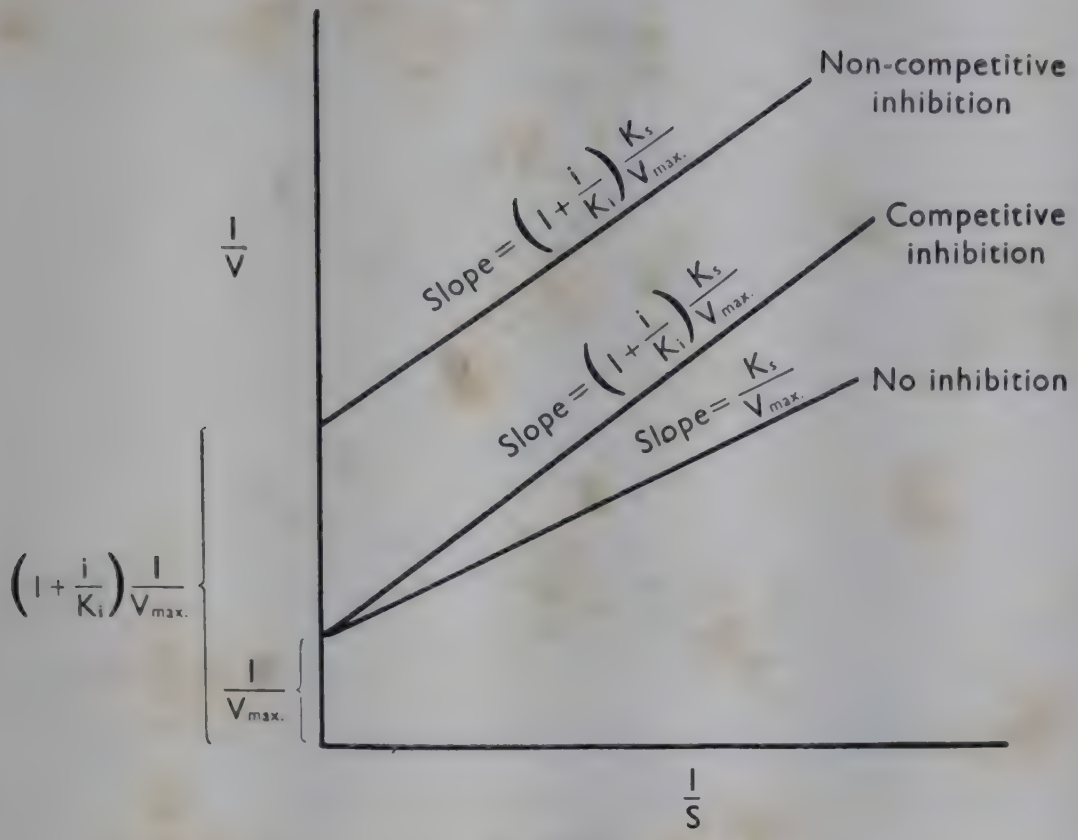


FIG. 6.6

Plot of $1/V_i$ against $1/s$ for competitive and non-competitive inhibition.

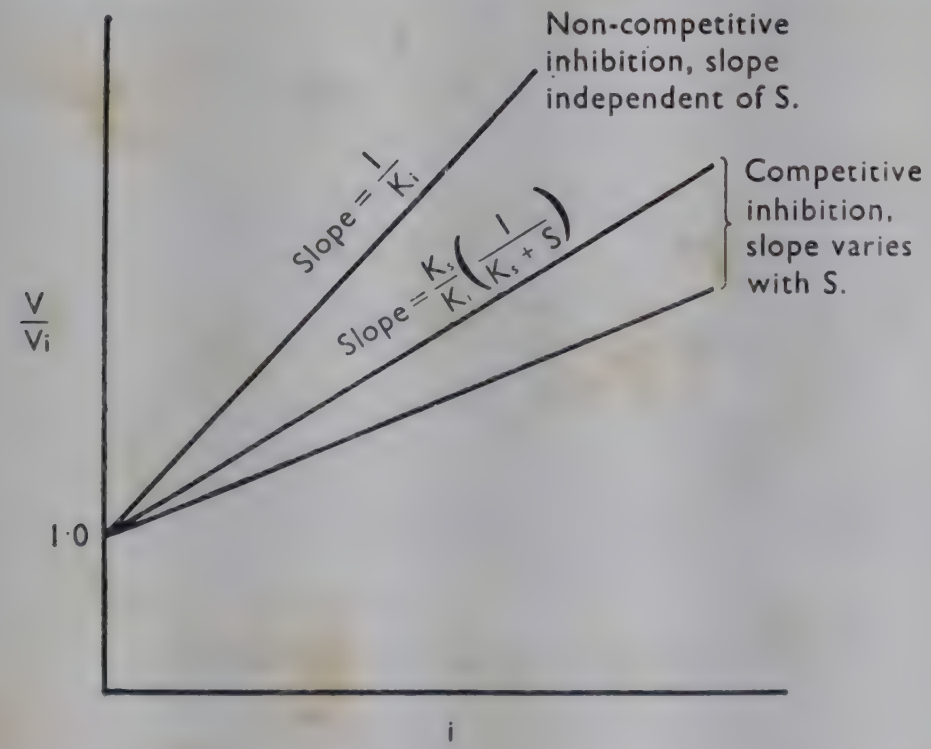


FIG. 6.7

Plot of V/V_i against i for competitive and non-competitive inhibitors. The slope of the former is dependent upon the substrate concentration s.

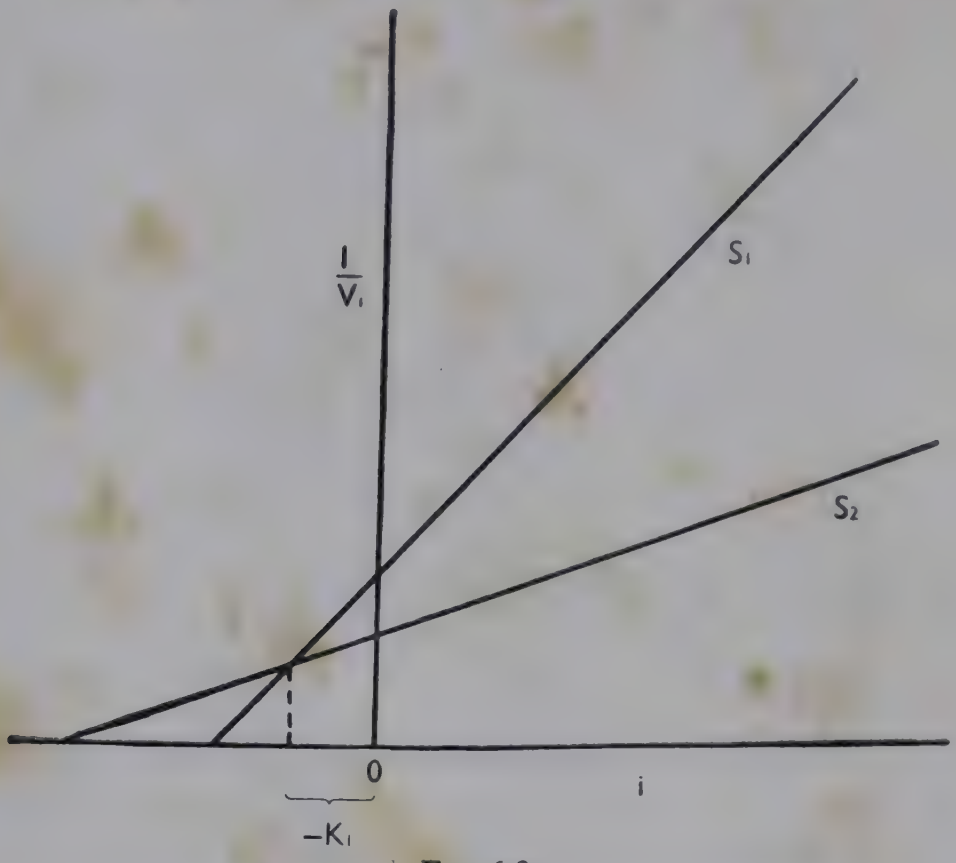


FIG. 6.8
Determination of K_i for a competitive inhibitor (DIXON, 1953).

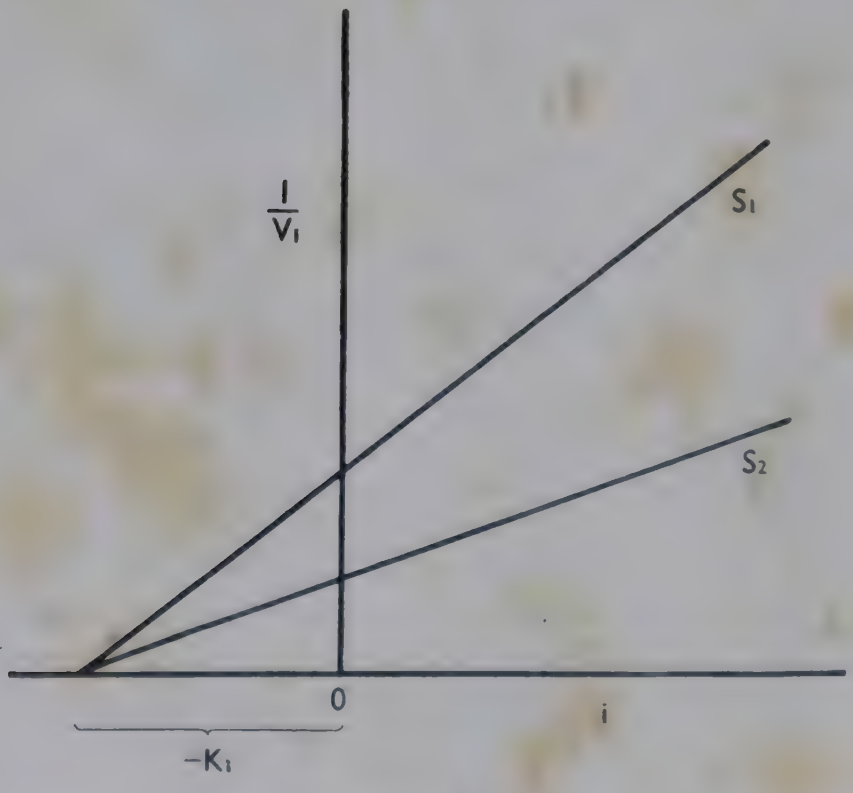


FIG. 6.9
Determination of K_i for a non-competitive inhibitor (DIXON, 1953).

The equation represents each line and at the point of intersection $1/V_i$ and i will be the same for both lines as also will be $V_{max.}$. Consequently

$$\left(1+\frac{i}{K_i}\right)\frac{K_s}{s_1}=\left(1+\frac{i}{K_i}\right)\frac{K_s}{s_2}.$$

This can be true only if either $s_1 = s_2$ or if $i = -K_i$, and since the former is not true, $i = -K_i$. When K_i has been evaluated, the same graph may be employed to determine K_s since each line cuts the abscissa at a value equal to $-K_i\left(\frac{s}{K_s}+1\right)$.

An alternative method is given by Dixon for use if K_s has already been determined by the Lineweaver–Burk plot in the absence of an inhibitor. In this case it is necessary only to determine the inhibitory effects at one substrate concentration. The lines intersect at a point giving K_i at a height of $1/V_{max.}$ and, as this value will already have been obtained in the original plot, a horizontal line drawn at a height of $1/V_{max.}$ will intersect the inhibitor line at a point equal to $-K_i$.

With non-competitive inhibitors in the plot of $1/V_i$ against i at two different substrate concentrations s_1 and s_2, the lines do not cross but they meet at a point on the abscissa which gives an intercept of $-K_i$. This is proved by putting $1/V_i = 0$ in equation 6.9, and the type of graph obtained is shown in Fig. 6.9.

Example 6.2.—It has been shown that the action of α-amylase on starch and on dextrins of high molecular weight is approximately 100 times as rapid as its action on the smaller dextrins formed subsequently. Schwimmer (1950) has investigated the kinetics of malt α-amylase action, including the effect of digestion products on the velocity of dextrinization of soluble starch by the enzyme, in an effort to elucidate this problem. His findings are given in the following table (p. 110).

The hydrolysis of starch proceeds in the order

Starch→α-dextrin→limit dextrin→maltose.

From the data provided it is possible to obtain, first, the value of K_s, the dissociation constant for the enzyme-substrate complex. This is obtained from the values of the relative hydrolysis velocities at different substrate concentrations in the absence of inhibitor by plotting $\frac{1}{s}$ against $\frac{1}{V}$. Thus

s	$\frac{1}{s}$	V	$\frac{1}{V}$
11·52	0·087	100	0·010
5·76	0·174	82	0·012
2·88	0·348	56	0·018
1·44	0·695	39	0·026

Inhibitor	*Inhibitor concentration* mg./ml.	*Substrate concentration*, mg./ml.			
		11·52	5·76	2·88	1·44
		Relative hydrolysis velocities			
None . .	0·00	100	82	56	39
Maltose . .	6·35	90	77	50	36
	12·70	82	66	46	31
	25·40	67	57	38	27
Limit dextrin .	6·68	102	80	44	31
	13·35	106	81	38	27
	26·70	100	84	29	19
α-Dextrin .	1·67	105	78	49	32
	3·34	100	76	45	28
	6·68	100	70	37	23

These reciprocals are plotted in Fig. 6.10, from which a value of K_S = 3·45 mg. per ml. is obtained. As the molecular weight of the starch is not given (it is

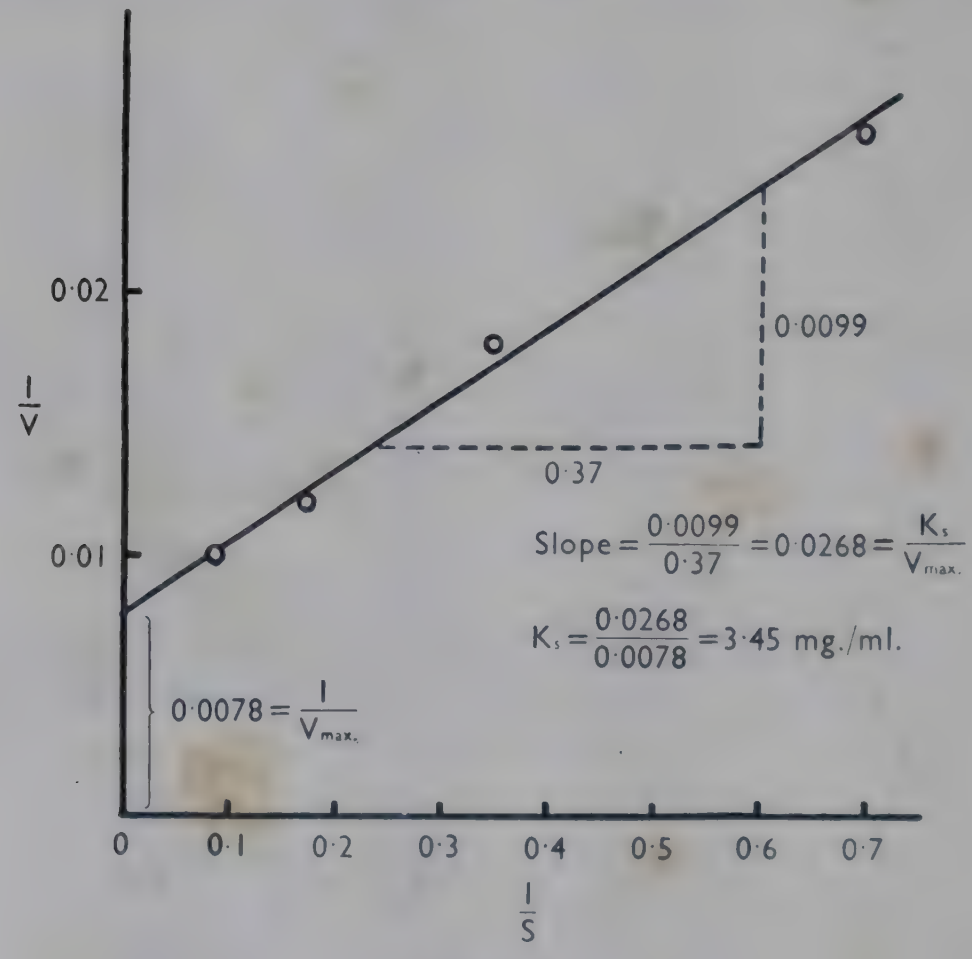


FIG. 6.10

still a matter of some dispute), the value for K_s cannot be given in terms of the customary moles per litre.

To determine the type of inhibition produced by maltose, limit dextrin and α-dextrin, and the K_i values involved, it is necessary to plot either $1/V$ against $1/s$ or, better in this instance, V/V_i against i.

As the velocities in the presence and absence of varying inhibitor concentrations are given, it is possible to calculate the V/V_i ratios for each substrate concentration. Thus, for maltose, the following figures are obtained:

Maltose concentration (mg./ml.)	*Substrate concentration, s,* (mg./ml.)											
	11·52			5·76			2·88			1·44		
i	V	V_i	$\frac{V}{V_i}$	V	V_i	$\frac{V}{V_i}$	V	V_i	$\frac{V}{V_i}$	V	V_i	$\frac{V}{V_i}$
0·00	100	—	—	82	—	—	56	—	—	39	—	—
6·35	—	90	1·11	—	77	1·065	—	50	1·12	—	36	1·08
12·70	—	82	1·20	—	66	1·24	—	46	1·22	—	31	1·26
25·40	—	67	1·50	—	57	1·44	—	38	1·48	—	27	1·44

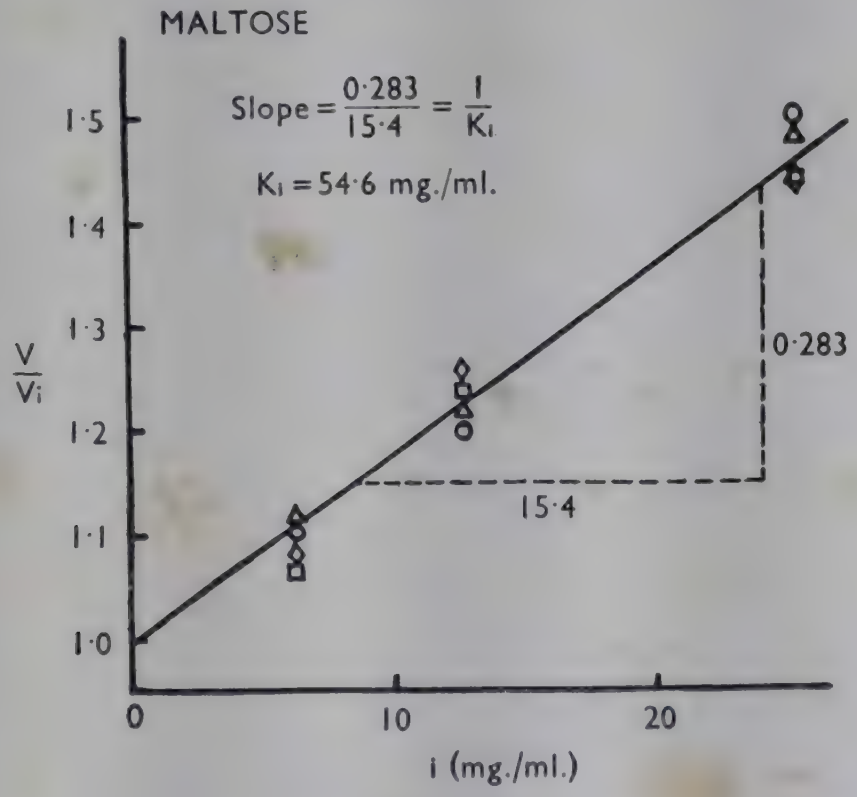


FIG. 6.11

Fig. 6.11 shows the plot of these V/V_i values against i, from which it will be observed that the sets of points for each inhibitor concentration are such that one straight line suffices for all, and there is an intercept on the ordinate of 1·0. This means that the slope of the line is independent of the substrate concentration s, and therefore the inhibition is non-competitive. Consequently the

slope of the line is equal to $1/K_i$ and this enables K_i to be evaluated. A figure of 54·6 mg. per ml. is obtained.

Exactly the same procedure is carried out for the limit dextrin and α-dextrin inhibitions, but, below, the full working out of the V/V_i values is omitted and the figures for them are simply recorded in the table.

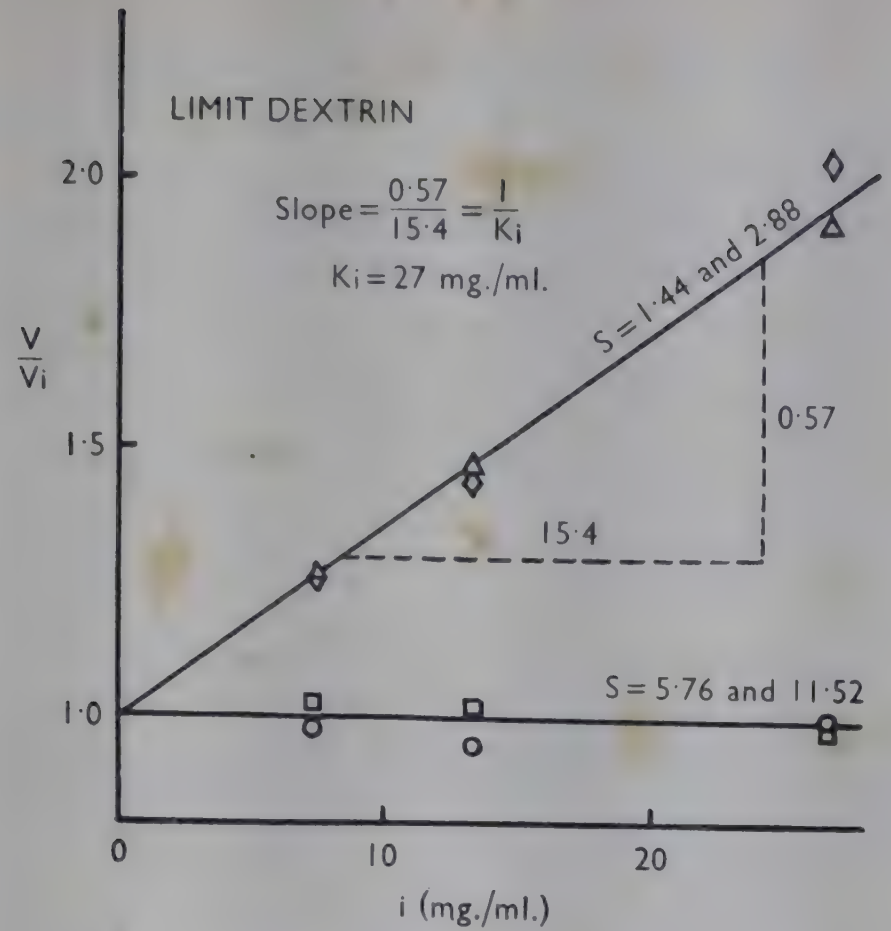
Inhibitor concentration (*i*) mg./ml.		*V/V_i values for substrate concentration* mg./ml.			
		11·52	5·76	2·88	1·44
Limit dextrin	6·68	0·98	1·03	1·27	1·26
	13·35	0·945	1·02	1·47	1·44
	26·70	1·00	0·98	1·93	2·05
α-Dextrin	1·67	0·95	1·05	1·11	1·22
	3·34	1·00	1·08	1·24	1·39
	6·68	1·00	1·17	1·51	1·70

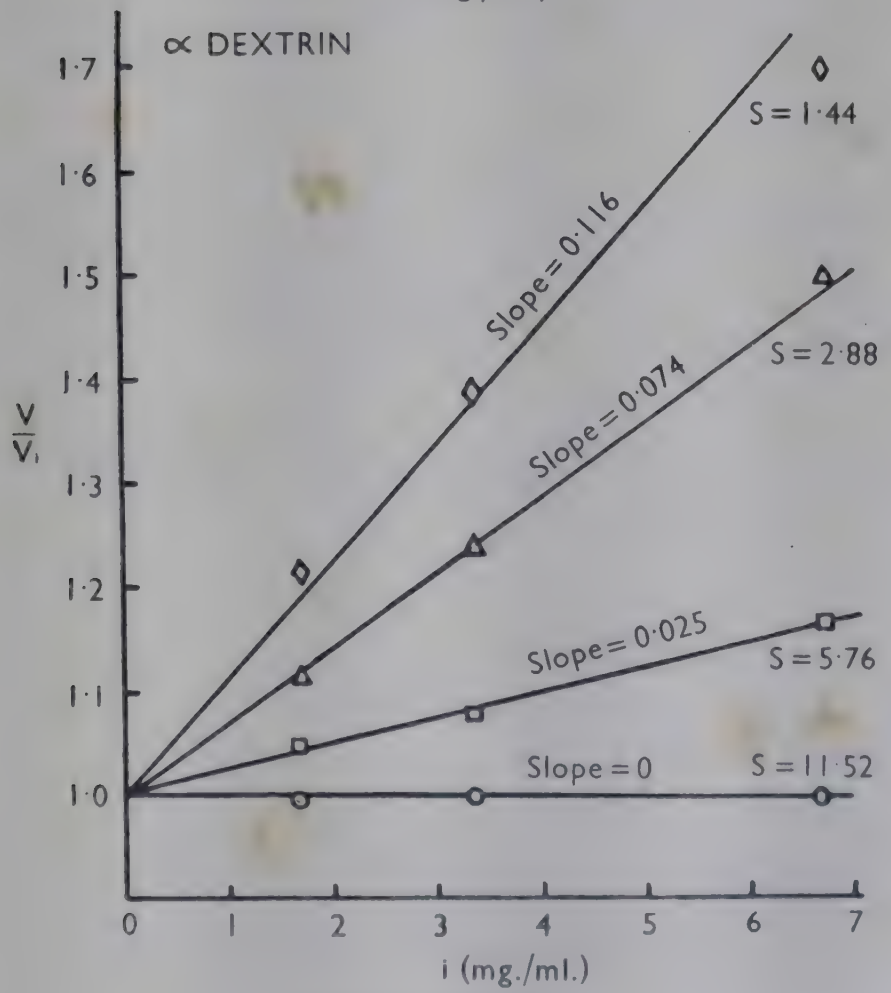
Figs. 6.12 and 6.13 show the plots of V/V_i against i. From Fig. 6.12 it will be noticed that at the highest substrate concentrations the limit dextrin does not inhibit over the concentration range employed, i.e. a straight line parallel to the i axis is obtained. At the two lowest substrate concentrations, however, inhibition does occur, but is the same in both cases; one straight line suffices for all the points. This indicates that the inhibition is non-competitive since the slope is obviously independent of substrate concentration in the range where inhibition occurs. Here again, K_i can be evaluated from the slope and a value of 27 mg. per ml. obtained. The behaviour of limit dextrin in inhibiting non-competitively at lower substrate concentrations and not at all at higher concentrations is unusual and no explanation has been offered to account for it. In Fig. 6.13 the results with α-dextrin are shown and a difference is at once noticeable. At the highest substrate concentration there is no inhibition, but with the other three concentrations inhibition does occur and the slopes of the lines increase with decreasing substrate concentration, i.e. increased inhibition. Here, therefore, the inhibition is competitive in nature. The values of K_i may be obtained for the different substrate concentrations by applying equation 6.7. The slope of the line where competitive inhibition occurs is equal to $\frac{K_s}{K_i}\left(\frac{1}{K_s + s}\right)$. The value of K_s was obtained previously from the plot of $1/V$ against $1/s$ and K_i therefore can be evaluated.

$$K_s = 3{\cdot}45 \text{ mg./ml.}$$

Substrate concentration, s mg./ml.	*Slope of line*	$\frac{K_s}{slope}$	$K_s + s$	$\frac{1}{K_s + s}$	K_i mg./ml.
1·44	0·116	29·75	4·89	0·205	6·1
2·88	0·074	46·6	6·33	0·158	7·4
5·76	0·025	138	9·21	0·108	14·9

These results show that the enzyme has less affinity for the inhibitor as the substrate concentration increases, i.e. K_i increasing as the substrate concentration increases.





FIGS. 6.12 and 6.13

Turnover Number.

Another very useful characteristic of an enzyme is its *turnover number*. This is defined as the number of molecules of substrate which are acted upon per minute by one molecule of enzyme when the enzyme is working at maximum rate, i.e. at its optimal pH and temperature. The determination of the turnover number (T.N.) clearly depends upon a knowledge of the molecular weight of the enzyme, and this is not known in many instances.

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PROBLEMS

6.1. The following rates of evolution of carbon dioxide were obtained in the decarboxylation of an amino acid at various concentrations.

Amino acid concentration millimolar	$\mu l.\ CO_2/5\ min.$
0·6	9·65
1·0	15·2
1·5	22·3
2·0	25·6
3·0	34·5
4·0	40·0

Calculate the Michaelis constant of the enzyme.

(Leeds General Studies Course, 1954.)

6.2. The deamination of both L-serine and L-threonine by *Escherichia coli* is believed to be catalysed by one and the same enzyme. The saturation curves for enzyme with substrate were obtained, using purified enzyme preparations, in the following manner:

Enzyme, substrate, co-factor additions (glutathione and adenosine-5-phosphate) and phosphate buffer pH 7·8, to give a final volume of 1 ml., were

incubated at 37° C. for 10 minutes and then the keto acid production determined in each case. Results obtained were as follows:

Substrate concentration mg./ml.	*Keto acid formed per 10 minutes* μmoles	
	L-serine substrate	*L-threonine substrate*
0·05	0·14	0·29
0·15	0·37	0·45
0·25	—	1·00
0·50	0·58	1·25
1·00	0·71	1·55
5·00	0·89	1·60

From these data determine the Michaelis constant for the enzyme-substrate complex with each substrate. What would you expect to happen if the enzyme were added to an equimolar mixture of L-serine and L-threonine?

(After Wood & Gunsalus (1949), *J. biol. Chem.*, **181**, 171.)

6.3. The effect of pH on the Michaelis constant of the 6-phosphogluconate dehydrogenase of rat liver has been investigated by Glock and McLean. Using glycylglycine buffer of pH 7·6 and 9·0, the activity of the dehydrogenase was measured spectrophotometrically by following the rate of reduction of triphosphopyridine nucleotide at 340 mμ. Results were obtained as follows:

Substrate concentration	*Increase in optical density in initial 5 minutes*	
$\times 10^4$ M	pH 7·6	pH 9·0
0·174	0·074	0·034
0·267	0·085	0·047
0·526	0·098	0·075
1·666	0·114	0·128
4·00	—	0·167

Determine at which pH value the enzyme has its greatest affinity for the substrate.

(After Glock & McLean (1953), *Biochem. J.*, **55**, 400.)

6.4. The following data were obtained in an experiment to determine the coenzyme saturation curve for a partially purified sample of tryptophanase, which catalyses the reaction

Tryptophan→Indole + Pyruvic acid + Ammonia.

The coenzyme used was barium pyridoxal phosphate. In each determination 2 mg. L-tryptophan were incubated with 0·03 ml. apoenzyme in 2 ml. buffer.

Indole formed in 10 minutes	*Barium pyridoxal phosphate present* μg./ml.
1·7	0·3
2·7	0·5
4·3	1·0
6·5	2·0
7·3	3·0
7·8	4·0
7·9	5·0
8·1	10·0

Calculate the dissociation constant of the pyridoxal phosphate-tryptophanase complex.

(After Wood, Gunsalus & Umbreit (1947), *J. biol. Chem.*, **170**, 313; Glasgow Honours Course Finals, 1953.)

6.5. The substrate saturation curve for tryptophanase (see Problem 6.4) has been determined and the following data obtained:

L-tryptophan concentration (mg. per 10 ml.)	*Indole produced per* 15 *min.* (μg. per 10 ml.)
0·25	23·9
0·50	28·6
1·0	37·7
1·5	41·0
2·0	43·2
3·0	43·2
4·0	41·9
5·0	44·4

From these figures obtain a value for the Michaelis constant for tryptophanase using the Lineweaver and Burk method of evaluation.

(After DAWES & HAPPOLD (1949), *Biochem. J.*, **44**, 349.)

6.6. Pig heart sarcosomes catalyse the oxidative phosphorylation of α-ketoglutaric acid according to the equation

$$\alpha\text{-ketoglutarate} + \tfrac{1}{2}O_2 + x\text{ADP} + xH_3PO_4 \rightarrow \text{succinate} + CO_2 + x\text{ATP} + xH_2O.$$

This reaction can be coupled to the phosphorylation of glucose to hexosemonophosphate (HMP) by the addition of glucose and yeast hexokinase, e.g.

$$x\text{ATP} + x\text{glucose} \rightarrow x\text{HMP} + x\text{ADP}.$$

ADP is thus regenerated and plays a catalytic role in the overall reaction, while the amount of phosphorylation occurring is measured by determining the increase in HMP concentration. The following information has been obtained regarding the effect of concentration of sarcosomes on the rate of oxidation of α-ketoglutarate and on phosphorylation of glucose to HMP.

	Sarcosomal protein (mg./ml.)			
	0·12	0·31	0·62	1·25
Oxygen uptake (μg. atoms)				
Without ADP	0·26	0·64	1·68	4·15
With excess ADP	0·70	1·76	3·56	6·26
$\triangle$*HMP* (μmoles)				
Without ADP	0	0·19	1·24	4·47
With excess ADP	1·82	4·36	8·46	15·8

Use the Michaelis-Menten equation to determine the average Michaelis constants of the endogenous ADP of the sarcosomes for oxidation and for phosphorylation. The sarcosome content of energy-rich phosphate (ADP) in μmoles is given by mg. sarcosomal protein $\times$ 0·018.

(After SLATER & HOLTON (1953), *Biochem. J.*, **55**, 530.)

6.7. What factors affect the rate of enzymic reaction? In an enzyme catalysed reaction the following data were obtained:

Velocity in moles/hour	*Substrate concentration* molar
0·1230	5·000
0·1212	2·500
0·1192	1·667
0·1177	1·250
0·1160	1·000
0·1143	0·833
0·1127	0·715
0·1111	0·625
0·1096	0·555
0·1081	0·500

Calculate the Michaelis constant of the enzyme and the maximum attainable reaction velocity.

(Leeds Honours Course Finals, 1954.)

6.8. The effect of temperature on the Michaelis constant of an enzyme has been investigated using the luciferin-luciferase system of the luminescent ostracod crustacean *Cypridina*.

The initial velocities of the reaction were followed by measuring photoelectrically the amount of light produced per unit time. Experimental conditions restricted the measurements to two temperatures viz. 15° and 22° C., and four series of experiments were performed, therefore, at each temperature to compensate for this limitation. Results from one such experiment are given below.

Ml. of luciferin solution per 20 ml. reaction mixture	*Initial velocity in millivolts/minute*	
	15°	22°
0·04	15	18
0·06	19	22
0·08	23	33
0·10	26	37
0·20	44	62
0·40	56	91
0·90	76	123

The workers estimated the concentration of the luciferin solution to be 8×10^{-5} M. Assuming this molarity, determine the effect of change of temperature on the Michaelis constant and comment on your results.

(After KAUZMANN, CHASE & BRIGHAM (1949), *Arch. Biochem.*, **24**, 281.)

6.9. Carbobenzoxy-L-glutamyl-L-tyrosine is hydrolysed by swine kidney pepsinase (cathepsin) in accordance with the equation:
Carbobenzoxy-L-glutamyl-L-tyrosine→carbobenzoxy-L-glutamic acid + L-tyrosine.

The reaction may be followed manometrically by the addition of tyrosine decarboxylase when CO_2 is evolved and tyramine formed,

i.e. L-tyrosine→tyramine + CO_2.

It was discovered that the carbobenzoxy-L-glutamic acid formed in the hydrolysis had an inhibitory effect on the progress of the reaction and hence caused a decrease in reaction rate. The nature of this inhibition was investigated by Frantz and Stephenson, who added carbobenzoxy-L-glutamic acid, at two different concentration levels, to the reaction mixture. Some of their data are recorded in the following table. From these figures demonstrate graphically that carbobenzoxy-L-glutamic acid is a competitive inhibitor of the cathepsin

reaction and obtain values for the Michaelis constant of cathepsin and also K_i, the dissociation constant of the enzyme-inhibitor complex.

Concentration of carbobenzoxy-L-glutamyl-L-tyrosine μmoles per ml.	*Concentration of carbobenzoxy-L-glutamic acid* μmoles per ml.	*Rate of CO_2 production* μmoles per ml.per min.
4·7	0·0	0·0434
4·7	7·58	0·0285
4·7	30·3	0·0133
6·5	0·0	0·0526
6·5	7·58	0·0357
6·5	30·3	0·0147
10·8	0·0	0·0713
10·8	7·58	0·0512
10·8	30·3	0·0266
30·3	0·0	0·1111
30·3	7·58	0·0909
30·3	30·3	0·0581

(After FRANTZ & STEPHENSON (1947), *J. biol. Chem.*, **169**, 359.)

6.10. The action of various compounds on crystalline carboxypeptidase activity has been studied by following their effects on the hydrolysis of L-carbobenzoxy-glycyl-D-phenylalanine. The course of the reaction was followed colorimetrically by use of ninhydrin and compounds investigated included phenylacetate, hydrocinnamate, phenylbutyrate and benzoate. Data obtained are recorded in the following table from which determine the types of inhibition involved.

Inhibitor	*Initial substrate concentration* M	*Reaction rate arbitrary units*
None	0·0713	166
	0·0581	142·6
	0·0384	111
	0·0285	111
	0·0125	66
0·002 M Hydrocinnamate	0·100	16
	0·050	11·1
	0·040	7·6
	0·025	5·55
0·002 M Phenylacetate	0·047	40
	0·033	33·3
	0·0166	18·1
0·002 M Phenylbutyrate	0·055	90·9
	0·040	57·1
	0·025	50·0
	0·0125	28·5
0·05 M Benzoate	0·100	40·8
	0·050	38·4
	0·025	33·3
	0·0175	30·3

(After ELKINS-KAUFMAN & NEURATH (1949), *J. biol. Chem.*, **178**, 645.)

6.11. The following data were obtained in an experiment to determine the turnover number of catalase:

The enzyme preparation, which was 85 per cent. pure, had a dry weight of 2·95 mg. per litre. Manometrically it was ascertained that 0·1 ml. of this preparation liberated 340 μl. oxygen, measured at N.T.P., from excess hydrogen peroxide in 10 minutes. The molecular weight of catalase may be taken as 225,000.

Use these data to calculate the turnover number of catalase. (The transition from α to β activity of the catalase preparation may be ignored and a uniform rate of oxygen evolution assumed.)

(Glasgow Honours Course Finals, 1952.)

6.12. The β-glucuronidase optimally active at pH 3·4 is inhibited by mucic acid. The following data were obtained in an experiment where the effect of variation of substrate concentration on reaction velocity was studied in the absence and presence of 0·0001 M mucate. The substrate was phenolphthalein glucuronide and the reaction velocity is expressed in μg. phenolphthalein liberated in 60 minutes at 37°. Determine the type of inhibitor and the enzyme-inhibitor dissociation constant.

Substrate concentration millimolar	*Reaction velocity*	
	No inhibitor	0·0001 M *mucate*
1	32·0	3·0
2	43·6	6·3
3	50·4	9·0
4	53·0	11·8
5	56·5	14·5
10	62·1	24·4

(Glasgow Honours Course Finals, 1954.)

6.13. The following data were obtained in an experiment to determine the affinity between tropine esterase and atropine sulphate as substrate. The reaction was followed manometrically in bicarbonate buffer by measuring the CO_2 evolution resulting from acid liberated by the hydrolysis of the atropine sulphate. Rabbit serum (2·5 per cent.) was the source of the enzyme and the reaction was carried out at 30° and pH 7·4.

Concentration of atropine sulphate		*CO_2 liberated in 100 min.*
Per cent.	M $\times 10^3$	mm.^{3}
0·250	7·4	28·5
0·100	3·0	28·0
0·050	1·5	28·0
0·025	0·74	26·6
0·0125	0·37	25·0
0·010	0·30	23·5
0·005	0·15	20·3

Determine the Michaelis constant of tropine esterase.

(After GLICK (1940), *J. biol. Chem.*, **134**, 617.)

6.14. Derive an expression relating the initial velocity of an enzyme reaction to the concentration of substrate present.

The enzymic decomposition of a substrate A is inhibited by compounds B and C. The following table gives initial velocities of decomposition of A (in micromoles/ml./min.) in the presence of B and C, separately, at concentrations stated (in millimoles/ml.). The amount of enzyme present was the same in every case.

(*a*)	Conc. of A:	10					
	Conc. of B:	0	0·5	2·0	3·0	4·0	8·0
	Velocity:	10	8·0	5·3	4·2	3·6	2·2
	Conc. of C:	0	1·0	2·0	3·0	4·0	5·0
	Velocity:	10	4·5	2·9	2·1	1·68	1·39
(*b*)	Conc. of A:	30					
	Conc. of B:	0	1·5	2·5	3·5	5·0	7·0
	Velocity:	20	11·8	9·0	7·8	6·2	4·8
	Conc. of C:	0	1·0	2·0	3·0	4·0	8·0
	Velocity:	20	15·4	11·8	10·0	8·4	5·3

What conclusions can you draw about the action of the inhibitors? Give your reasons.

(Leeds Honours Course Finals, 1955.)

6.15. The influence of ATP concentration on the rate of dephosphorylation of ATP by myosin, which catalyses the reaction

$$\text{ATP} \rightarrow \text{ADP} + \text{inorganic phosphate}$$

has been studied at 25° and pH 7·0. The following data were obtained:

Velocity of reaction	*ATP concentration*
μmoles inorganic phosphate produced/litre/sec.	μM
0·067	7·5
0·095	12·5
0·119	20·0
0·149	32·5
0·185	62·5
0·191	155·0
0·195	320·0

Determine the Michaelis constant of myosin.

(After OUELLET, LAIDLER & MORALES (1952), *Arch. Biochem. Biophys.*, **39**, 37.)

6.16. The citridesmolase enzyme of *Aerobacter aerogenes* carries out the reaction:

$$\text{Citrate} \rightarrow \text{Oxaloacetate} + \text{Acetate}$$

and requires magnesium as a cofactor.

The progress of the reaction may be followed by determining the keto acid produced. The following data were obtained in experiments to investigate the effect of calcium ions on the reaction, and at the times of analysis keto acid was being produced linearly with time. The reaction mixture consisted of

phosphate buffer pH 7·0, enzyme and citrate ; magnesium and calcium ions were added to various concentrations.

1. Concentration of Mg^{2+} in reaction mixture, 8·35 mM. Incubation time, 100 sec.

ml. $CaCl_2$ (0·025 M) added to 3 ml. reaction mixture :	0	0·1	0·2	0·3	0·4
μmoles/litre of keto acid produced :	1175	965	725	615	540

2. Concentration of Mg^{2+} in reaction mixture, 2·88 mM. Incubation time, 120 sec.

ml. $CaCl_2$ (0·025 M) added to 3 ml. reaction mixture :	0	0·1	0·2	0·3	0·4
μmoles/litre of keto acid produced :	1020	676	480	370	316

3. Concentration of Mg^{2+} in reaction mixture, 0·96 mM. Incubation time, 180 sec.

ml. $CaCl_2$ (0·025 M) added to 3 ml. reaction mixture :	0	0·1	0·2	0·3	0·4
μmoles/litre of keto acid produced :	865	420	286	226	166

4. Concentration of Mg^{2+} in reaction mixture, 0·32 mM. Incubation time, 210 sec.

ml. $CaCl_2$ (0·025 M) added to 3 ml. reaction mixture :	0	0·1	0·2	0·3
μmoles/litre of keto acid produced :	590	220	130	105

Use these data to determine

(*a*) the Michaelis constant of citridesmolase for Mg^{2+},

(*b*) the type of inhibition produced by Ca^{2+},

(*c*) the dissociation constant of the enzyme-inhibitor complex.

(After DAGLEY & DAWES (1955), *Biochim. biophys. Acta*, **17**, 177.)

CHAPTER VII

PHOTOMETRIC ANALYSIS

Biochemists make considerable use of photometric methods of analysis. Nowadays it is accepted as a general principle that optical methods should be used wherever possible, always provided they furnish the desired degree of accuracy, in preference to other types of analysis, on account of the rapidity, simplicity and sensitivity of measurement which modern apparatus permits. The latter is of particular importance, since it enables very small quantities of metabolites and other substances of vital significance in biological material to be determined.

Colorimeters and spectrophotometers measure the amount of light absorbed by coloured or colourless solutions, turbidimeters and nephelometers measure the light scattered by suspensions, and fluorimeters determine the fluorescence produced by absorbed light. Flame photometers analyse the alkali and alkaline earth metal constituents of biological material by means of their emission spectra. In addition to these, infra-red spectrometers are now finding increasing use as biochemical equipment. Ultraviolet and visible absorption spectra afford information concerning proteins, nucleic acids, coenzymes, cytochromes and other pigments, and fluorescent spectra are used for the analysis of thiamine and riboflavin.

Theoretical Background.

The energy of a molecule may be divided into three main categories, namely electronic, vibrational and rotational. The energy is quantized in discrete levels, as represented diagrammatically for the case of a simple diatomic molecule in Fig. 7.1. The energy difference between electronic levels is very much greater than that between the vibrational levels, and rotational levels are the closest of all. When some change within the molecule entails transition from one energy level (E_1) to another (E_2), radiation is either emitted or absorbed in accordance with the equation:

$$E_1 - E_2 = h\nu, \quad . \quad . \quad . \quad (7.1)$$

where h is Planck's constant ($6{\cdot}62 \times 10^{-27}$ erg sec.) and ν the frequency of the radiation emitted or absorbed. If E_1 is greater than E_2, the radiation is emitted, but if the transition should be

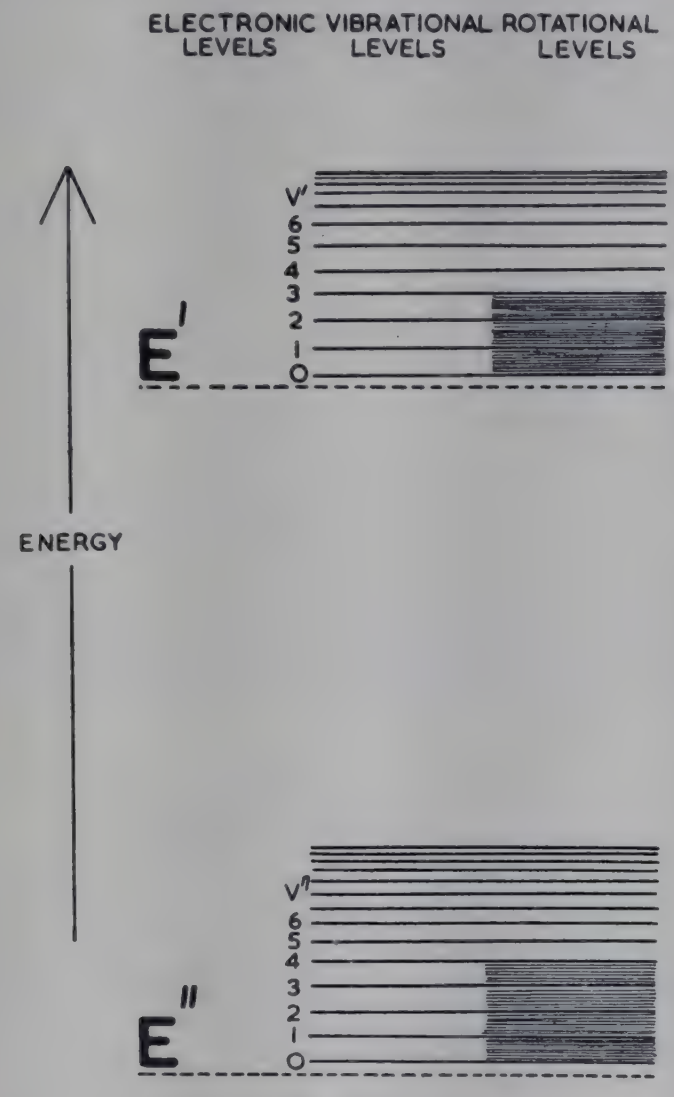

FIG. 7.1

Energy levels of a diatomic molecule. E' and E'' are upper and lower electronic energy levels respectively. V' and V'' are vibrational quantum numbers. Rotational quantum numbers have been omitted for clarity.

from E_2 to E_1 then the radiation is absorbed. Since frequency and wavelength (λ) are related by the expression

$$c = \nu\lambda,$$

where c is the velocity of light (ca. 3×10^{10} cm./sec.), small energy changes correspond to low frequencies or long wavelengths. Thus the position of a spectral line or band may be expressed in terms of wavelength or frequency. It will be appreciated from

the foregoing that the region of the spectrum in which a molecule emits or absorbs radiation is dependent solely on the energy levels within that molecule.

Frequency is measured in units of sec.$^{-1}$, which is somewhat inconvenient because the numbers involved are so large. Occasionally it is expressed in fresnels (10^{12} vibrations/sec.), but the common practice is to use waves per centimetre or wave

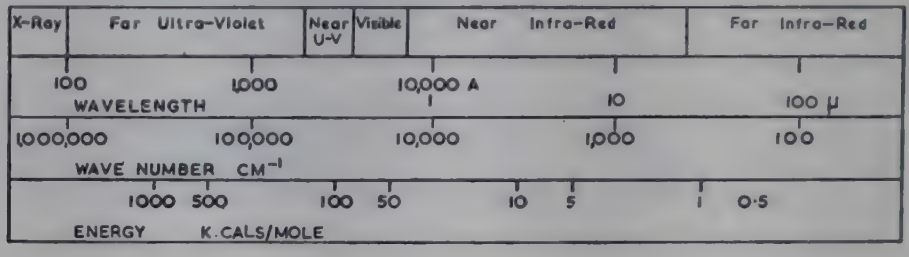

FIG. 7.2

The main regions of the electromagnetic spectrum.

numbers (cm.$^{-1}$). Wave numbers, which are denoted by $\bar{\nu}$, are directly proportional to energies and can be readily expressed as frequencies since a constant factor, the velocity of light, is involved. Thus

$$\bar{\nu} \text{ cm.}^{-1} = \frac{10^8}{\lambda \text{ in Ångstroms}} = \frac{\nu}{c}.$$

Wavelength (λ) is expressed in either Ångstroms (Å), microns (μ) or millimicrons (mμ), although the latter finds widest application in the biochemical literature. They are related as follows :

$$1 \text{ Å} = 10^{-8}\text{cm.} \qquad 1 \text{ m}\mu = 10^{-7}\text{cm.} \qquad 1 \mu = 10^{-4}\text{cm.}$$

The main regions of the electromagnetic spectrum are shown in Fig. 7.2. The division into regions is rather arbitrary and is mainly decided by the type of experimental technique required. In this discussion attention will be confined to the near ultraviolet and visible regions, which are most commonly employed by biochemists, and where transitions between electronic, vibrational and rotational levels occur. This is not intended to imply that the other regions are without interest. Crystal analysis by X-ray techniques is of value in connexion with problems of molecular structure, the far ultraviolet is the region of characteristic absorption by simple molecules, and the near infra-red is the region of vibrational and rotational (but not electronic) transitions,

where it is possible to assign absorption maxima to particular groups in the molecule and thus elucidate problems of molecular structure.

When light is absorbed, the molecule responsible for the absorption is excited to a higher energy state, in which it usually remains for approximately 10^{-8} second. The excited molecule can lose its energy in several ways, namely

1. by dissociation of the molecule,
2. as heat,
3. as light of different wavelengths (fluorescence, Raman effect),
4. as light of different wavelengths after a time lag (phosphorescence).

A graph showing the amount of light absorption as a function of the wavelength is known as an *absorption spectrum*, and, because it depends on chemical structure, a different spectrum is obtained with each different structure. A means of detecting different chemical structures is thereby afforded and absorption spectra have been likened to chemical 'fingerprints'. It is perhaps worth emphasizing that, although similarity in chemical structure can be ascertained by absorption spectra, absolute identity with a given compound cannot be established on these grounds alone. Nevertheless, it is an exceptionally valuable investigational method and determinations of absorption spectra play an important role in modern biochemistry.

When light falls on any body or solution, part is reflected, part is absorbed with the effects already discussed, and part is transmitted. The relation

$$I_0 = I_r + I_a + I_t$$

may be written, where I_0 is the intensity of the incident light and I_r, I_a and I_t are respectively the intensities of light reflected, absorbed and transmitted. Fresnel demonstrated that the amount of light reflected is given by the expression

$$I_r = \left(\frac{n-1}{n+1}\right)^2 I_0 = kI_0 \quad . \quad . \quad (7.2)$$

where n is the refractive index of the medium. Aqueous solutions are generally used in biochemistry, in which case k becomes negligible and I_r may be neglected.

The Laws of Lambert and Beer.

Colorimetry and spectrophotometry are based on the laws of Lambert and Beer. Lambert studied the amount of monochromatic light absorbed by a body and derived the following relationship:

$$I = I_0 10^{-Kl} \quad . \quad . \quad . \quad . \quad (7.3)$$

where I_0 and I are the intensities of the incident and transmitted light respectively, l is the thickness of the absorbing material and K is the *extinction coefficient*,[1] defined as the reciprocal of the thickness of medium (in cm.) required to weaken the light to one tenth of its incident intensity.

Now $Kl = E$, which is known as the *extinction* or *optical density*.

Hence $$E = \log \frac{I_0}{I} \quad . \quad . \quad . \quad . \quad (7.4)$$

The percentage transmission of light, T, is equal to $100I/I_0$ and it follows that the optical density is related to T by the expression

$$E = \log 100 - \log T = 2 - \log T \quad . \quad . \quad (7.5)$$

Beer studied the influence of the concentration of a coloured substance in solution upon the monochromatic light transmission and found the same relationship between transmission and concentration that Lambert found between transmission and thickness of layer, e.g. 1 cm. depth of 1 M solution absorbs the same amount of light as 10 cm. depth of 0·1 M solution. This is expressed in the equation

$$I = I_0 10^{-\epsilon cl} \quad . \quad . \quad . \quad . \quad (7.6)$$

where c is the molar concentration of the substance, l the depth of the solution and ϵ is the *molar extinction coefficient* of the solute for the particular wavelength in question. Comparing equations 7.3 and 7.6, it will be seen that

$$\epsilon c = K \quad . \quad . \quad . \quad . \quad (7.7)$$

In the case of many natural products the molecular weight may not be known with certainty and it is customary to express the

[1] A table listing the symbols used in spectrophotometry is given on page 136.

concentration in terms of grams per ml. Here the *specific extinction coefficient*, $\epsilon_{spec.}$, is used and

$$\epsilon_{spec.} C = K \quad . \quad . \quad . \quad (7.8)$$

where C is the concentration of the substance in g./ml.

Deviations from Beer's Law.—Beer's Law is always considered valid, but where deviations from it are encountered in experimental data another reason is sought such as chemical change, e.g. hydration, dissociation, complex formation, etc. For instance, certain salts form complexes the colours of which are different from those of the simple compounds, and as the concentration of the complex form decreases with dilution, Beer's Law does not hold. Furthermore, suspensions do not obey the Law.

Apparent deviations from Beer's Law may be instrumental in origin, as when too wide a slit width is used in spectrophotometry and the light is not monochromatic.

The Absorption Ratio

The ratio of the concentration of a coloured substance in g./ml. to its extinction coefficient is a characteristic constant for each substance and depends only upon the wavelength and the nature of the solvent employed. This value is known as the *absorption ratio* and is denoted by A.

$$A = \frac{C}{K} \quad . \quad . \quad . \quad . \quad (7.9)$$

It will be appreciated that except in regions where the absorption does not alter with change in wavelength there will be a different absorption ratio for each wavelength of light used. However, the normal practical procedure is to make the measurement in a region of maximum absorption.

Knowledge of the absorption ratio is of value because it enables the concentration of a substance to be determined and also permits its qualitative detection. Since for a given wavelength and solvent A depends solely on the nature of the pigment, it follows that the same pigment in two different solutions will have the same value of A if dissolved in the same solvent and measured at the same wavelength. The absorption ratio may also be taken as a criterion of purity in the isolation of a pigment. If the pigment is

contaminated with colourless materials, the value of A will be increased, and hence the smaller the absorption ratio the greater the purity of the pigment.

The absorption ratio is determined by obtaining the extinction coefficient of the substance at various concentrations and at a wavelength corresponding to the peak of an absorption band. Provided Beer's Law holds, the values of A obtained should be the same, within the limits of experimental error, for all concentrations. Since it is essential to use monochromatic light for such determinations, spectrophotometric measurement (described later) is essential.

Example 7.1.—An aqueous solution containing 5 mg. of a coloured compound per 50 ml. gave an optical density of 0·835 when measured through 2·5 mm. depth of liquid at 546 mμ. Calculate the absorption ratio.

The absorption ratio $A = \frac{C}{K}$ and $E = Kl$,

where C is the concentration in g./ml. and l the depth in cm.

Hence $C = \frac{0{\cdot}005}{50} = 0{\cdot}0001$ g./ml. and $l = 0{\cdot}25$ cm.

Therefore $A = \frac{0{\cdot}0001 \times 0{\cdot}25}{0{\cdot}835} = \underline{2{\cdot}994 \times 10^{-5}}$.

Colorimetry.

Consider two solutions of a coloured compound having concentrations c_1 and c_2. These are placed in a colorimeter, an instrument which, in one of its forms (the Duboscq colorimeter), permits the thickness of the layers of liquid to be changed and measured and which allows the amounts of transmitted light to be compared. When the system is optically balanced, i.e. when the two layers have the same colour intensity, then the two beams of transmitted light have the same intensity and $I_1 = I_2$ (see Fig. 7.3). Furthermore, since I_0 is the same for each,

$$\epsilon c_1 l_1 = \epsilon c_2 l_2,$$

and under the conditions where Beer's Law holds

$$c_1 l_1 = c_2 l_2.$$

Consequently a colorimeter permits the determination of the concentration of a coloured substance by comparison with a solution of known concentration provided Beer's Law holds and

the system is optically balanced. Let c_x be the unknown concentration, then,

$$c_x = \frac{cl}{l_x} \quad . \quad . \quad . \quad . \quad (7.10)$$

l and l_x are determined experimentally by the colorimeter, c is known and hence c_x may be obtained.

Example 7.2.—A solution treated with Nessler's reagent and containing 20 μg. ammonia per 10 ml. was placed in a Duboscq colorimeter and set at a depth of 15 mm. A second solution, similarly treated and containing an unknown amount of ammonia, was matched against the former and the mean of ten readings was 12·5 mm.

Let c be the concentration of the unknown, then

$$12{\cdot}5 \times c = 15 \times 20$$

and

$$c = 24 \ \mu\text{g. } NH_3 \text{ per 10 ml.}$$

The light source for a colorimeter is ordinary white light, but this does not alter the validity of the method provided no attempt is made to match colours of different hue but of apparently equal intensity. The only remedy for this latter situation is the use of

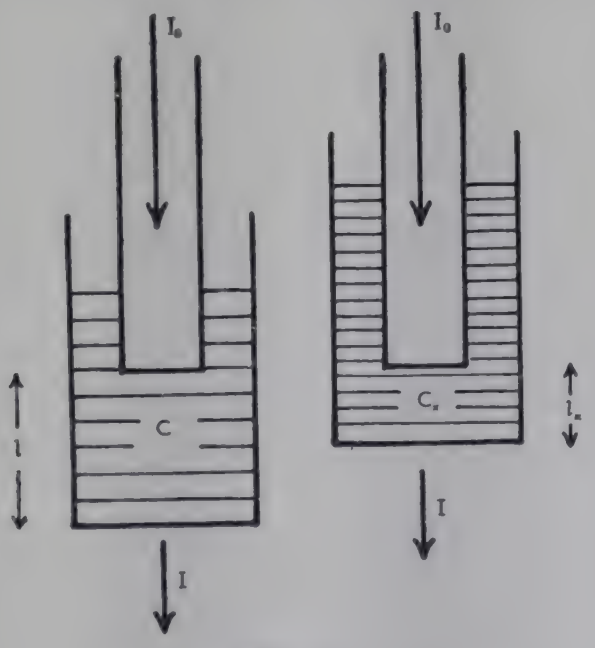

FIG. 7.3

Principle of the Duboscq colorimeter, which permits adjustment of length of light path through the liquids so that the intensities of transmitted light are equal.

monochromatic light, although by the use of suitable filters it is possible to select light of a fairly narrow waveband.

The type of colorimeter described above is the visual one, but

photoelectric instruments are now widely used. These colorimeters usually consist of a light source from which light passes through a suitable filter, an adjustable diaphragm and a tube containing the solution under investigation, to impinge finally on a photocell. The current generated is fed to a milliammeter usually calibrated to give an optical density reading directly. In use the blank solution, e.g. solvent, is placed in position and the diaphragm adjusted to give a zero scale reading; standard and unknown solutions are then, in turn, placed in the light path and the scale readings observed. By simple proportion the concentration of the unknown solution may be calculated.

Example 7.3.—Determinations of lactic acid by the Barker and Summerson method were carried out by photoelectric colorimeter using a green filter. A standard solution containing 30 μg. lactic acid per determination gave a reading of 36·5 on the scale and two unknown solutions gave values of 16 and 45. The amount of lactic acid in the unknowns is therefore:

(*a*) $$\frac{30 \times 16}{36{\cdot}5} = \underline{13{\cdot}2}\ \mu\text{g.}$$

(*b*) $$\frac{30 \times 45}{36{\cdot}5} = 36{\cdot}98 \approx \underline{37}\mu\text{g.}$$

Calibration Curves.—Where colorimetric determinations are being carried out routinely with photoelectric instruments, the best method of analysis is to prepare a calibration curve covering a suitable range of concentration. Optical density or scale readings are plotted against substrate concentration and then, on all future occasions, by reference to this graph the concentration corresponding to any observed colorimeter reading may be ascertained without the use of standard solutions (Fig. 7.4(*a*)). With such a graph the range over which Beer's Law holds (i.e. the linear portion) is readily appreciated and it also permits analyses to be extended outside this range provided the deviation from linearity is not too great. Errors which might otherwise arise through using the proportionality calculation (Example 7.2) in a region where Beer's Law does not apply are thereby eliminated. This latter point emphasizes the general principle that when standard solutions are used for colorimetric comparison they should be as near the optical density of the unknown solution as possible.

It has been noted that Beer's Law does not apply to suspensions. However, for turbidimetric work, it is possible to prepare for suspensions a colorimeter calibration which usually displays

curvature throughout. The density of bacterial suspensions is often measured in this manner (Fig. 7.4(*b*)).

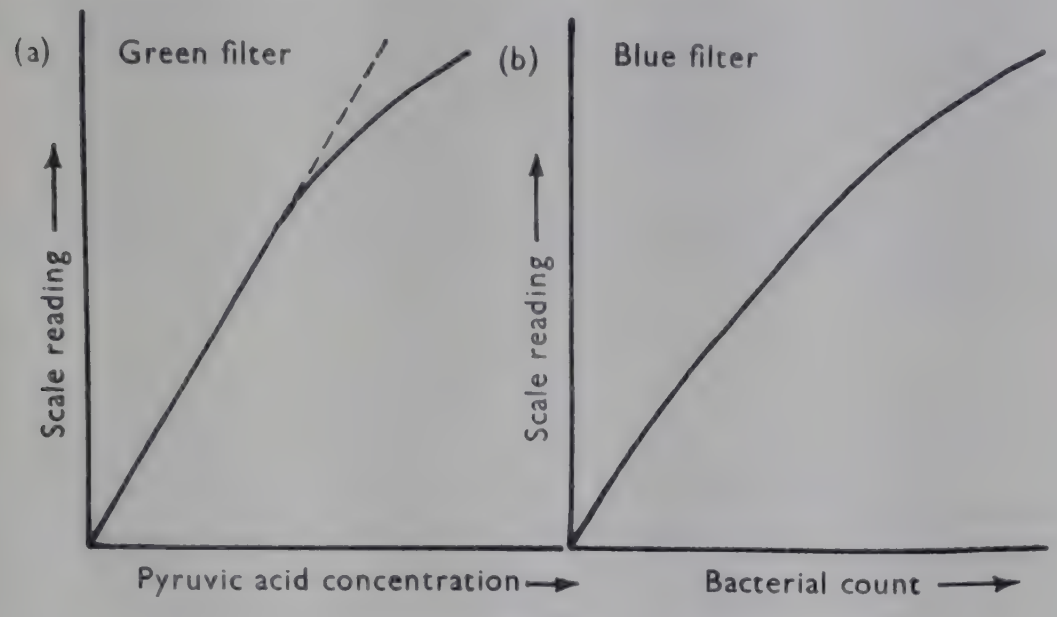

FIG. 7.4

Colorimeter calibration curves for
(*a*) estimation of pyruvic acid as alkaline 2:4-dinitrophenylhydrazone and
(*b*) estimation of bacterial suspension density.

Spectrophotometry.

A spectrophotometer is an instrument which uses monochromatic light and enables the extinction, E, to be measured for various solutions at different wavelengths. The desired wavelength is selected by means of a monochromator (either prism or diffraction grating) in conjunction with an adjustable slit, which controls the spectral width, and the solution is placed in a cuvette of known light path l (usually 1 cm.). The spectrophotometer measures E (i.e. $\log I_0/I$) directly, and hence, with known concentration c, the molar extinction coefficient can be determined. When E is known for a given wavelength, the concentrations of unknown solutions may be measured. If Beer's Law is obeyed, the concentration is simply proportional to the measured optical density. The notation usually employed is

$$E_e^c(\lambda)$$

to denote the extinction coefficient at concentration c with light path of length l and at wavelength λ. Modified slightly, this same notation can be applied to the absorption of natural products,

whether substances of uncertain molecular weight or even mixtures, by expressing c as a percentage (w/v) of solute.

Example 7.4.—If a 0·5 cm. layer of 0·2 per cent. (w/v) solution transmits one-tenth of the incident light at 300 mμ, then $E = 1{\cdot}0$, since $E = \log I_0/I = \log 10$.

The measurement may be specified as

$$E^{0{\cdot}2\%\ (\mathrm{w/v})}_{0{\cdot}5\ \mathrm{cm.}}\ 300\ \mathrm{m}\mu = 1{\cdot}0.$$

For convenience of comparison 1 per cent. (w/v) solution and 1 cm. light path are usually taken as standard and the previous measurement is converted to this basis as follows:

$$E^{1\%\ (\mathrm{w/v})}_{1\ \mathrm{cm.}}\ 300\ \mathrm{m}\mu = \frac{1{\cdot}0}{0{\cdot}5 \times 0{\cdot}2} = 10.$$

Mixtures of several absorbing substances can be analysed in certain cases. Where the absorption bands of the individual substances of the mixture do not overlap, the analysis is simple and straightforward. Wavelengths are selected where each component in turn displays an absorption adequate for measurement and the other components display negligible absorption. Thus one measurement of optical density at each chosen wavelength suffices to determine each individual substance in the mixture. An important application of this method is the determination of tyrosine and tryptophan in unhydrolysed proteins. Since tryptophan is easily destroyed by hydrolytic procedures, the spectroscopic technique affords the most reliable method of analysis for this amino acid.

Where the absorption bands overlap and there are n components of the mixture, the absorption must be measured at n wavelengths, chosen so that at each the specific extinction coefficient of one component is much greater than the others. The solution of n simultaneous equations enables the concentrations to be determined. To illustrate the method consider a mixture containing three components, X, Y and Z, of concentrations C_X, C_Y and C_Z. At three chosen wavelengths λ_1, λ_2 and λ_3 the specific extinction coefficients are respectively x_1, x_2, x_3, y_1, y_2, y_3 and z_1, z_2, z_3. Assuming Beer's Law to be obeyed, the extinction coefficient of the mixture at each wavelength is the sum of the values for the individual components. Thus

$$K_1 = C_X x_1 + C_Y y_1 + C_Z z_1$$
$$K_2 = C_X x_2 + C_Y y_2 + C_Z z_2$$
$$K_3 = C_X x_3 + C_Y y_3 + C_Z z_3$$

and the three simultaneous equations are solved for C_X, C_Y and C_Z.

THE ESTIMATION OF TWO PIGMENTS BY HÜFNER'S QUOTIENTS.—If two pigments are simultaneously present in one solution it is possible to estimate their proportions spectrophotometrically by a method devised by Hüfner for determining haemoglobin and oxyhaemoglobin in mixtures. Consider pigments M and N, which do not influence one another optically, dissolved in the same solvent. The optical density at any wavelength will be the sum of the individual optical densities, i.e.

$$E = E_M + E_N.$$

For each pigment the quotient of the optical densities at wavelengths λ_1 and λ_2 will have a characteristic value which is determined, say, *a* for M and *b* for N. Thus, for mixtures of M and N the quotient may have all values between *a* and *b* and the more of M is present the closer will the quotient lie to *a* and the more of N the closer will it lie to *b*. Consequently determination of the optical density of a mixture of M and N at wavelengths λ_1 and λ_2 will permit calculation of the composition of the mixture. Clearly the wavelengths selected should be such that the two pigments display the largest possible difference in quotient values. Let the mixture contain *x* per cent. of M.

At λ_1

$$E(\lambda_1) = E_M + E_N$$

$$= \frac{x}{A_{M_1}} + \frac{100 - x}{A_{N_1}} \quad . \quad . \quad (7.11)$$

where A_{M_1} and A_{N_1} are the absorption ratios for M and N respectively at wavelength λ_1. Similarly at λ_2

$$E(\lambda_2) = \frac{x}{A_{M_2}} + \frac{100 - x}{A_{N_2}} \quad . \quad . \quad (7.12)$$

Hence the Hüfner quotient will be

$$\frac{E(\lambda_1)}{E(\lambda_2)} = \frac{\dfrac{x}{A_{M_1}} + \dfrac{100 - x}{A_{N_1}}}{\dfrac{x}{A_{M_2}} + \dfrac{100 - x}{A_{N_2}}} \quad . \quad . \quad (7.13)$$

which can be solved for x. Hüfner did this for all possible values of $E(\lambda_1)/E(\lambda_2)$ for mixtures of haemoglobin and oxyhaemoglobin, carboxyhaemoglobin and oxyhaemoglobin, and methaemoglobin and oxyhaemoglobin respectively, and tabulated his results. Thus, knowledge of the quotient permits the percentage composition of the mixture to be read from the table. Alternatively, calibration curves may be prepared showing the Hüfner quotient as a function of the percentage composition of the mixture.

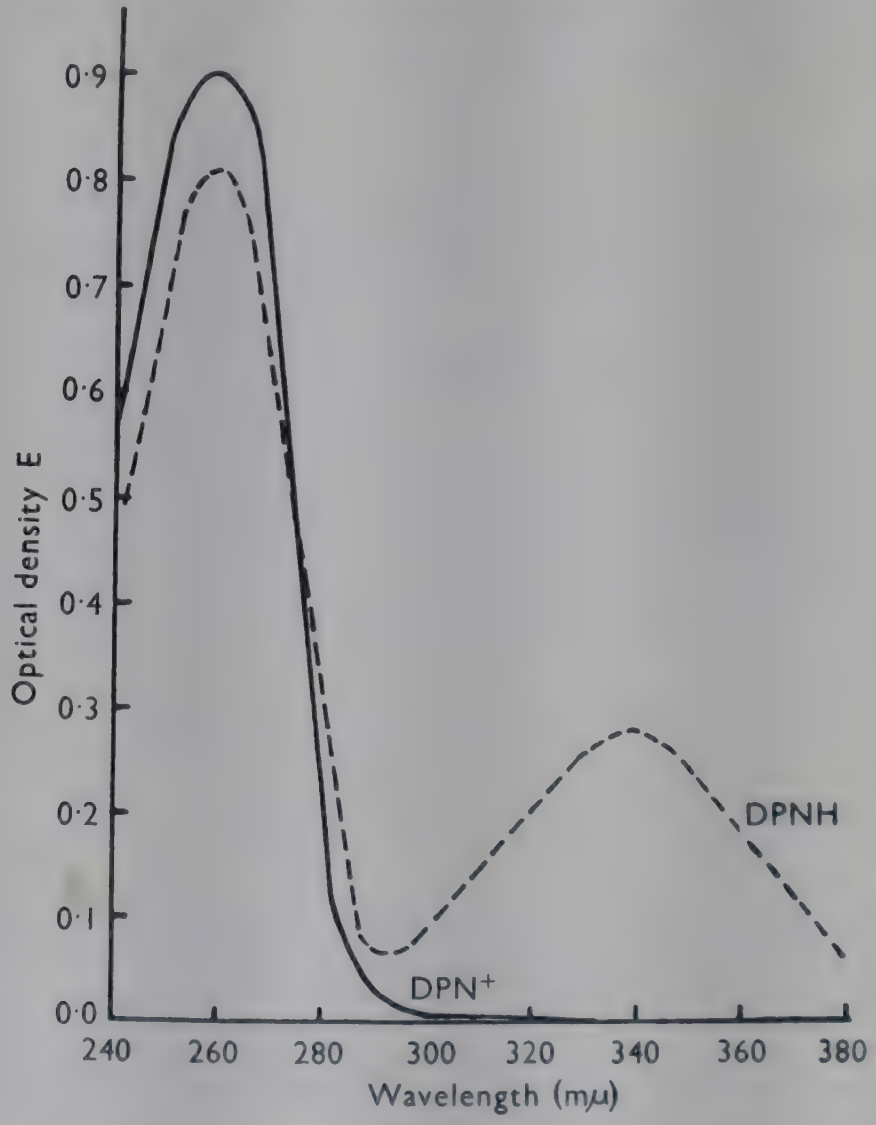

FIG. 7.5

Absorption spectra of oxidized (DPN^+) and reduced (DPNH) diphosphopyridine nucleotide.

ABSORPTION SPECTRA.—The measurement of absorption spectra, to which reference has already been made, is carried out by means of a spectrophotometer, and in Fig. 7.5 are shown the spectra of oxidized and reduced diphosphopyridine nucleotide (DPN^+ and

DPNH respectively). It will be observed that both exhibit a major absorption peak at 260 mμ, characteristic of their nucleotide structure, but the spectrum of the reduced form shows a smaller absorption band with a maximum at 340 mμ whereas the oxidized form does not. This difference forms the basis of a widely used method for following oxidation-reduction reactions involving DPN^+ (or triphosphopyridine nucleotide, TPN^+, which displays similar spectra in oxidized and reduced forms). By carrying out the reaction in a spectrophotometer cuvette with wavelength selected for 340 mμ, the reduction of DPN^+ may be observed by the increase in optical density that occurs as the reaction proceeds. Conversely, oxidation of DPNH results in decrease of optical density at 340 mμ. Thus, in the case of the DPN-linked malic dehydrogenase system

$$\text{Malate}'' + DPN^+ \rightleftharpoons \text{Oxaloacetate}'' + DPNH + H^+$$

the optical density at 340 mμ increases as the reaction proceeds from left to right and decreases as the reverse reaction takes place. Examples of such experiments will be found in some of the problems of Chapter IV.

Nephelometry.

Nephelometry enables the turbidity of a suspension to be measured by means of the Tyndall effect, i.e. the light scattered by the particles in suspension. The nephelometric method has been used by T. W. Richards to determine the true end-point of the silver nitrate-sodium chloride titration in high precision work for determining the atomic weight of silver. It is also widely used for determining the density of bacterial suspensions. One type of nephelometer passes a beam of light vertically through an iris diaphragm to the hemispherical base of the test tube, which when containing a clear liquid functions as a condenser lens and produces a parallel beam passing centrally up the tube. The focusing action of the tube base is decreased by a turbid solution which also scatters light from the vertical column of liquid. This scattered light is received by a number of photocells which are so arranged round the base of the test tube that they do not receive light directly from the lamp. The current generated is fed to a milliammeter which indicates a measure of the turbidity of the solution. The method is advantageous for following bacterial

growth because there is no interference with the contents of the growth tube.

Fluorimetry.

Substances which fluoresce in ultraviolet light may be determined by the techniques of fluorimetry, and two important members of the B group of vitamins can be assayed easily and rapidly in this way. Riboflavin fluoresces and thiamine can be oxidized by alkaline potassium ferricyanide to form thiochrome, which fluoresces in ultraviolet light.

Fluorimeters are so arranged that ultraviolet light enters the solution and a portion of the fluorescent radiation produced at an angle normal to the irradiating beam enters a phototube which is protected by a filter from the exciting ultraviolet light. Because the amount of fluorescent radiation is relatively small it is usually necessary to have a reflecting galvanometer and a scale, which is set up at a distance of 2 metres from the galvanometer. Calibration curves are prepared by using standard solutions, and unknown solutions may then be determined.

Flame Photometry.

Flame photometry depends on the emission spectra obtained when alkali or alkaline earth metals are subjected to heat excitation in a gas flame. Light emitted by the flame is focused through suitable optical filters on to a barrier layer photocell and the current generated is taken through a calibrated potentiometer to operate a taut suspension galvanometer. The instrument is calibrated with standard metal solutions, and full-scale deflections

TABLE 7.1

Terms used in Absorption Spectrophotometry

Term	*Symbol*	*Significance*
Extinction or optical density .	E	$E = \log I_0/I$
Transmission (percentage) .	T	$T = 100I/I_0$
Extinction coefficient . .	K	$I = I_0 10^{-Kl}$
Molar extinction coefficient .	ϵ	$I = I_0 10^{-\epsilon cl}$; $\epsilon = K/c_{\text{molar}}$
Specific extinction coefficient .	$\epsilon_{\text{spec.}}$	$\epsilon_{\text{spec.}} = K/C_{\text{g./ml.}}$
Absorption ratio . . .	A	$A = C_{\text{g./ml.}}/K = 1/\epsilon_{\text{spec.}}$

of the galvanometer may be obtained with as little as 5 parts per million (p.p.m.) sodium and 10 p.p.m. postassium. In flame photometry there is the possibility of considerable interference by ions other than those being determined.

SUGGESTED READING

GILLAM, A. E., & STERN, E. S. (1954). *An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry*. London: Arnold Ltd.

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PROBLEMS

7.1. What percentage of the incident light is reflected when a light beam is directed perpendicularly to the surface of benzene, ethyl acetate and acetone? The refractive indices of these compounds are respectively 1·5011, 1·3722 and 1·3589.

7.2. Calculate the optical densities corresponding to the following values of the percentage of transmitted light ($100I/I_0$):

(*a*) 95; (*b*) 88; (*c*) 71; (*d*) 50; (*e*) 17·5; (*f*) 1·0.

7.3. Protein concentration has been estimated from the intensity of biuret colours. What experiments would you carry out to ascertain the conditions of maximum accuracy in a given case? At a certain wavelength a density reading of 0·16 is obtained from a 0·18 per cent. solution in a cell with depth of 1 cm. Calculate an extinction coefficient.

(Leeds Honours Course Finals, 1954.)

7.4. Pyruvic acid may be determined colorimetrically by conversion to its 2:4 dinitrophenylhydrazone followed by the addition of alkali. To calibrate a photoelectric colorimeter the reaction was carried out on solutions containing various amounts of pyruvic acid. The following results were obtained:

Pyruvic acid μg./3 ml.	20	40	60	80	100	125	150	175
Scale reading	0·130	0·257	0·390	0·515	0·628	0·750	0·855	0·940

A series of unknown solutions of pyruvic acid was analysed by this method together with a standard solution containing 50 μg. pyruvic acid per 3 ml. Scale readings recorded were (*a*) 0·280, (*b*) 0·555, (*c*) 0·690, (*d*) 0·773 and (*e*) 0·910 for the unknowns and 0·325 for the standard. What percentage of error is introduced in the analysis by using the proportional method of calculation based on the 50 μg. standard instead of the previously established calibration curve?

7.5. A solution of a compound (0·001 M) was placed in a spectrophotometer cuvette of light path 1·05 cm. and the percentage light transmission recorded was 18·4 at 470 mμ. Determine the molar extinction coefficient. If the molecular weight of the compound was 215, what is the specific extinction coefficient?

7.6. The molar extinction coefficient of reduced triphosphopyridine nucleotide (TPNH) at 340 mμ is $6{\cdot}22 \times 10^3$ cm². 3 ml. of solution containing 0·2 μmoles TPNH were placed in a cuvette of 1·05 cm. light path. Calculate the percentage light transmission of this sample at 340 mμ.

7.7. 3 ml. of a solution of partially reduced diphosphopyridine nucleotide were placed in a 1 cm. spectrophotometer cuvette and the optical density determined at 340 and 260 mμ. The values obtained were 0·207 and 0·900 respectively. Calculate the molar concentrations of oxidized and reduced forms of the nucleotide.

The molar extinction coefficient of DPNH at 340 mμ is $6{\cdot}22 \times 10^3$ cm.² and the molar extinction coefficients of DPN^+ and DPNH may be assumed to have the same value of $18{\cdot}0 \times 10^3$ cm². at 260 mμ.

7.8. The following data were obtained by Heilmeyer in order to calculate an absorption ratio for bilirubin. The bilirubin was dried to constant weight before making up into solution in chloroform.

Bilirubin concentration mg./100 ml. $CHCl_3$	*Optical density at* 450 mμ	*Depth of liquid layer* mm.
2	0·491	2·56
5	0·503	1·05
7	0·711	1·05
10	0·995	1·05

Determine the absorption ratio for each solution of bilirubin and ascertain whether or not Beer's Law is valid for these solutions.

(Data from HEILMEYER (1943), *Spectrophotometry in Medicine*, Trs. Jordan & Tippell. London: Adam Hilger Ltd.)

7.9. A mixture of *ortho*, *meta* and *para* cresols dissolved in cyclohexane may be analysed spectrophotometrically in straightforward manner because each exhibits an absorption band in a region where absorption due to the other cresols is negligible. The absorption maxima occur at 752, 776 and 815 $cm.^{-1}$ for *ortho*, *meta* and *para* cresols respectively. To test the validity of Beer's Law for solutions of cresols each was made up in cyclohexane at a series of concentrations and the optical densities measured. Data obtained are recorded below.

ortho		*meta*		*para*	
Concentration g./100 ml.	*E* 752 $cm.^{-1}$	*Concentration* g./100 ml.	*E* 776 $cm.^{-1}$	*Concentration* g./100 ml.	*E* 815 $cm.^{-1}$
0·25	0·120	0·60	0·115	0·50	0·09
0·50	0·235	1·15	0·220	1·00	0·20
1·00	0·465	2·35	0·460	2·10	0·405
2·00	0·820			3·15	0·60

An unknown mixture of the three cresols in cyclohexane was analysed and the percentage light absorption at 752, 776 and 815 $cm.^{-1}$ was 14·5, 50 and 41 respectively. Determine the concentration of each cresol and the percentage composition of the mixture.

(After WHIFFEN & THOMPSON (1945), *J. chem. Soc.*, 268.)

7.10. The determination of the proportion of carboxyhaemoglobin and oxyhaemoglobin in mixtures of the two by means of characteristic extinction

coefficient quotients was worked out by Heilmeyer and Krebs. The wavelengths selected were 576 and 560 mμ, corresponding respectively to the first absorption maximum and minimum of oxyhaemoglobin. Their results are tabulated below.

Percentage of carboxyhaemoglobin	*Quotient* K_{576}/K_{560}	*Percentage of carboxyhaemoglobin*	*Quotient* K_{576}/K_{560}
0	1·725	55	1·190
5	1·666	60	1·153
10	1·611	65	1·115
15	1·558	70	1·078
20	1·507	75	1·042
25	1·457	80	1·007
30	1·410	85	0·974
35	1·363	90	0·940
40	1·318	95	0·908
45	1·275	100	0·877
50	1·233		

Use these data to construct a calibration curve for the determination. What percentage of oxyhaemoglobin is present in mixtures giving extinction coefficient quotients (576/560) of 1·580, 1·220 and 0·930? Suggest how it might prove possible to determine the absolute concentrations of oxyhaemoglobin and carboxyhaemoglobin in such mixtures and indicate what additional information would be required.

(After HEILMEYER & KREBS, quoted by HEILMEYER (1943), in *Spectrophotometry in Medicine*, Trs. Jordan & Tippell. London: Adam Hilger Ltd.)

7.11. The following data are taken from experiments to determine the extinction coefficients of reduced di- and triphosphopyridine nucleotides. The sample of nucleotide used need not be pure if an enzyme reaction is used with pure substrate and the pyridine nucleotide is in excess. Under suitable conditions the change in absorption is due to the reaction of a quantity of nucleotide equivalent to the added substrate. The reactions selected were:

Pyruvic acid + $DPNH_2 \rightleftharpoons$ Lactic acid + DPN

Acetaldehyde + $DPNH_2 \rightleftharpoons$ Ethanol + DPN

d-Isocitric acid + TPN $\rightleftharpoons$ α-Ketoglutaric acid + $TPNH_2$ + CO_2

and under the conditions selected proceeded from left to right virtually to completion. The lactic dehydrogenase of rabbit muscle also reacts with TPN, although at a much slower rate, and this nucleotide was also used in the system. The cells were of 1 cm. light path and the observed optical densities at 340 mμ were as follows:

System	*Optical Density*		*Concentration of substrate* μmoles/cm.^{3} × 10^3
	*Initial**	*Final*	
1. Pyruvate – $DPNH_2$	0·648	0·190	73·3
2. Pyruvate – $TPNH_2$	0·494	0·212	45·0
3. Acetaldehyde – $DPNH_2$	0·620	0·485	22·1
4. Isocitrate – TPN	0·167	0·526	60·6

* Corrected for dilution due to substrate addition.

Calculate the molar extinction coefficient for each experiment.

(After HORECKER & KORNBERG (1948), *J. biol. Chem.*, **175**, 385.)

7.12. The following data were obtained in an experiment to calibrate a nephelometer for bacterial turbidity measurements. A suspension of *Aerobacter aerogenes* containing 610×10^6 cells per ml. was diluted over a tenfold range and the original suspension used as a reference standard. With this suspension in the nephelometer the light intensity was adjusted to give a scale reading of 100 and the other suspensions were in turn placed in the instrument and the readings taken. These are given below.

Cell numbers millions/ml.	610	549	488	427	366	305	264	183	122	61
Galvanometer reading	100	91·5	81·5	73·0	66·0	58·5	47·0	42·0	32·0	23·5

Plot the resultant calibration curve and comment on the behaviour of this curve at low bacterial densities.

CHAPTER VIII

MANOMETRY

MANOMETRIC techniques are of considerable importance for the investigation of biochemical reactions in which gas exchanges are directly or indirectly involved. For instance the progress of oxidation, decarboxylation and photosynthetic reactions may be followed directly by the gas consumption or evolution concerned, whereas fermentation reactions and hydrolyses, during the course of which acid is produced, can also be measured by carrying out the reaction in bicarbonate buffer and recording the carbon dioxide evolution.

The advantage of manometric techniques lies in their extreme versatility and they have been put to many varied uses in furthering biochemical knowledge. At the present time the Warburg constant volume manometer and the Barcroft-Haldane differential instrument find most favour, and of these the former is more widely used. In this chapter attention will be confined to the Warburg apparatus, and the reader is referred to the excellent books by Dixon (1951) and Umbreit, Burris and Stauffer (1949) for details concerning other types of manometer.

The Warburg Constant Volume Manometer.

The principle involved is that if the volume of a gas is held constant, at constant temperature, any changes in the quantity of gas may be measured by changes in pressure. The instrument is illustrated in Fig. 8.1 and consists of a flask attached to the manometer by means of a ground glass joint. The flask may have one or more side bulbs which permit the addition of the substrate or reagents at intervals as required; it has also a centre well to which alkali is added when carbon dioxide is to be absorbed. The manometer fluid is contained in a reservoir and its level can be adjusted by means of the screw clamp. The tap permits the flask to be opened to the air. When assembled, the apparatus is fitted on a shaking device attached to a thermostat and so arranged that the flask is completely submerged in the bath. Accurate temperature control

is necessary. In operation the level of fluid in the closed limb of the manometer is always adjusted to the zero mark and the level in the open limb recorded. This observed pressure difference (in millimetres) when multiplied by a constant, which must be determined for each flask and manometer, gives the quantity of gas evolved or absorbed. To enable the constant to be calculated it is necessary to know the gas volume of the flask, the volume of fluid in it, the gas being exchanged, the temperature, and the density of the manometer fluid. The way in which the constant is derived is given below.

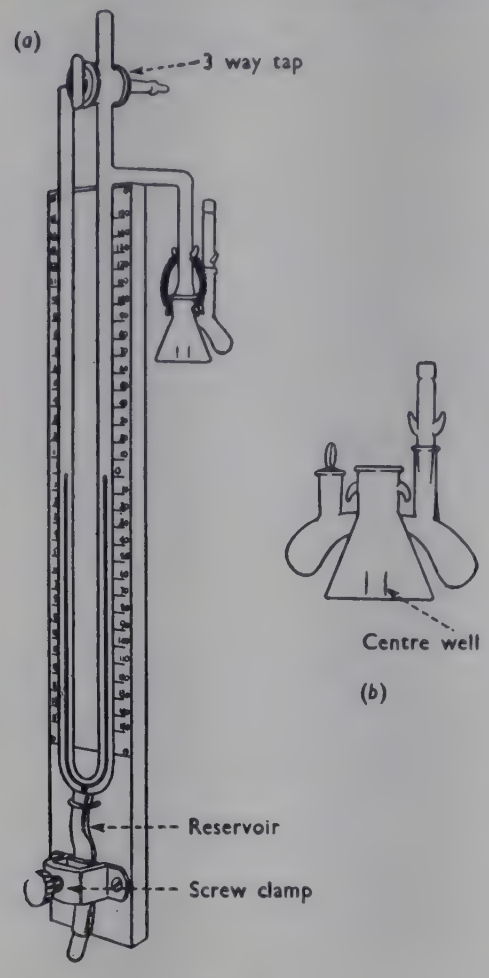


FIG. 8.1

(*a*) Warburg constant volume manometer with single side arm flask attached.

(*b*) Enlarged view of double side arm flask.

The customary convention is to regard all quantities of gas evolved as positive and all quantities absorbed as negative. Gas evolution is indicated by a rise of liquid in the open limb of the manometer when readings will therefore be positive. In the following treatment all quantities of gas are expressed in μl. of dry gas at N.T.P.

Let x = the quantity of gas evolved in μl. at N.T.P. (if the gas is absorbed x will be negative).

V_g = the volume of the gas space in the vessel (including the connecting and manometer tubes down to the zero mark).

h = the observed manometer reading in mm.

V_f = the volume of fluid in the vessel.

P = the initial pressure in the vessel of the gas being determined.

P_0 = normal pressure (760 mm. Hg) in mm. of manometric fluid. If D is the density of the fluid,

$$P_0 = \frac{760 \times 13{\cdot}60}{D} \text{ (density of Hg} = 13{\cdot}60 \text{ at } 0^\circ \text{ C.)}.$$

T = the absolute temperature of the thermostat.

p = the vapour pressure of water at temperature T.

α = the solubility of the evolved gas in the liquid in the vessel (expressed as μl. gas at N.T.P. dissolved in 1 μl. liquid when in equilibrium with a partial pressure of the gas equal to P_0).

Initial amount of gas in gas space

$$= V_g \frac{273(P - p)}{TP_0}$$

and initial amount of dissolved gas

$$= V_f \frac{\alpha(P - p)}{P_0}.$$

The final amount of gas in gas space

$$= V_g \frac{273(P - p + h)}{TP_0}$$

and the final amount of dissolved gas

$$= V_f \frac{\alpha(P - p + h)}{P_0}.$$

But the final total amount of gas present will be the sum of the amount initially present and the amount x evolved.

Thus

$$\left(V_g \frac{273}{T} + V_f\alpha\right)\frac{P - p + h}{P_0} = \left(\frac{V_g 273}{T} + V_f\alpha\right)\frac{P - p}{P_0} + x,$$

whence

$$x = h\left[\frac{\frac{V_g 273}{T} + V_f\alpha}{P_0}\right] \quad . \quad . \quad . \quad (8.1)$$

From this equation it will be appreciated that the expression within the brackets remains constant for a given gas with a given manometer and flask provided the liquid volume and the temperature are unchanged. This term is known as the flask 'constant' (k) of the apparatus and, as already mentioned, multiplication of the pressure difference h by it gives the quantity in μl. of dry gas at N.T.P. evolved. Thus

$$x = hk \quad . \quad . \quad . \quad . \quad (8.2)$$

CALIBRATION.—The value of the constant may be determined, i.e. the instrument calibrated, by one of three methods:

1. Calculation by use of equation 8.1.

2. Addition or withdrawal of a measured amount of gas from the flask by means of a graduated pipette and noting the change in manometer reading (Münzer and Neumann method).

3. By liberating or absorbing a known amount of gas in the manometer vessel by means of a chemical reaction, e.g. liberation of CO_2 from bicarbonate by acid.

THERMOBAROMETER.—Since the Warburg manometer has one end of the manometer tube open to the air, readings will be affected by slight changes in barometric pressure or in temperature of the thermostat which may occur during the course of an experiment. To eliminate errors arising in this way, an additional manometer containing water is always set up to function as a thermobarometer. Whenever readings are taken, the thermobarometer is also read and its value subtracted from those of the other manometers; changes in external conditions are thereby compensated.

Example 8.1.—A Warburg manometer was calibrated by the bicarbonate method. 2 ml. of 2 N hydrochloric acid were placed in the flask and 1 ml. of 10^{-2} M sodium bicarbonate added from the side arm after equilibration. When gas evolution ceased the manometer reading was +129 mm. and the thermobarometer registered −3·5 mm. Determine the flask constant.

The reaction employed is

$$NaHCO_3 + HCl = NaCl + CO_2 + H_2O$$

and each mole of bicarbonate yields 1 mole of CO_2.

$$1 \text{ ml. } 10^{-2} \text{ M } NaHCO_3 \equiv \frac{0{\cdot}01}{1000} \text{ moles}$$

$$\equiv 10 \ \mu\text{moles.}$$

Hence 10 μmoles of CO_2 are evolved by the excess acid and

$$x_{CO_2} = 22{\cdot}4 \times 10 = 224\ \mu l.$$

The true manometer reading is $+129 - (-3{\cdot}5) = 132{\cdot}5$ mm.

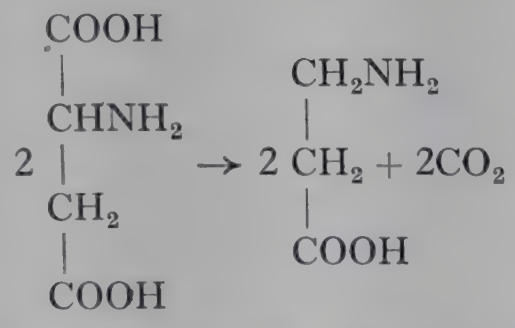
Hence the flask constant $k_{CO_2} = \dfrac{224}{132{\cdot}5} = \underline{1{\cdot}62}$

Units.

The basis of all manometric measurements is the gram-molecular volume (G.M.V.), which is the volume occupied by one gram-molecule of any gas at normal temperature and pressure and has the value of 22·4 litres. In manometry very much smaller quantities are usually dealt with, and the following table defines the units employed :

1 mole of any gas at N.T.P. occupies	22·4 litres
1 millimole (m-mole) = 10^{-3}mole occupies	22·4 millilitres (ml.)
1 micromole (μmole) = 10^{-6}mole occupies	22·4 microlitres (μl.)

For example, the decarboxylation of 2 μmoles of aspartic acid results in the formation of 2 μmoles of CO_2 and β-alanine respectively



Hence the volume of CO_2 evolved will be $2 \times 22{\cdot}4 = 44{\cdot}8$ μl. Specific bacterial amino acid decarboxylases have been used by Gale for the quantitative analysis of amino acids in amounts that are far too small to be analysed by classical chemical methods.

Respiratory Quotients

The respiratory quotient or R.Q. is defined as the relationship between the volume of carbon dioxide produced and the volume of oxygen consumed in respiration

$$\text{i.e. R.Q.} = \frac{CO_2 \text{ produced}}{O_2 \text{ consumed}}.$$

The R.Q. gives an indication of the nature of the metabolism

which is taking place. Complete oxidation of carbohydrate gives a value of 1·0 whereas an average fat yields a value of approximately 0·7 and an average protein a value of 0·8. Oxidation of a mixture of substrates results in an intermediate value.

Example 8.2.—What is the R.Q. when (*a*) glucose and (*b*) triolein ($C_{57}H_{104}O_6$) is completely oxidized?

(*a*) $C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$ R.Q. = 6/6 = 1·0.

(*b*) $C_{57}H_{104}O_6 + 80O_2 = 57CO_2 + 52H_2O$ R.Q. = 57/80 = 0·71.

Metabolic Quotients

To express the rate of oxygen uptake by a particular tissue or micro-organism values known as *metabolic quotients* are used. They take various forms and are denoted by the symbol Q_X, where X is the metabolite being measured. Thus Q_{O_2} is defined as the μl. oxygen taken up per milligram dry weight of biological material per hour. It has a negative value. Where the metabolite is a solid or liquid it is for the purpose of this definition regarded as a gas at N.T.P. so that 1 μmole is equivalent to 22·4 μl. Other examples, where a different tissue basis is employed, are as follows and it will be noted that the tissue basis is given in brackets:

$$Q_{O_2}(N) = \mu\text{l. } O_2\text{/mg. tissue nitrogen/hour}$$

$$Q_{O_2}(C) = \mu\text{l. } O_2\text{/mg. tissue carbon/hour}$$

If the atmosphere used in the manometer is other than air, it is also specified as a suffix to the quotient and the general form of the quotient then becomes

$$Q^{\text{gas atmosphere}}_{\text{metabolite measured}} \text{ (tissue basis)}$$

For instance, if oxygen uptake is measured in an atmosphere of pure oxygen and expressed on the basis of tissue nitrogen, the metabolic quotient will be denoted by $Q^{O_2}_{O_2}(N)$ whereas a fermentation reaction, carried out in an atmosphere of nitrogen and followed by CO_2 evolution from bicarbonate buffer, on the basis of tissue carbon will be given as $Q^{N_2}_{CO_2}(C)$. It is important, of course, that the weight basis used should represent as nearly as possible 'active' cell material, since inclusion of inert material will make very misleading the comparison of Q_X values with those of other tissues or organisms.

Example 8.3.—A bacterial suspension, respiring in the absence of substrate, absorbed 32μl. of oxygen in the initial 15 minutes. If the Warburg flask contained 12 mg. dry weight of organisms what is the Q_{O_2} value based on this initial rate?

$$Q_{O_2} = \mu l.\ O_2/mg.\ dry\ weight/hour$$
$$= \frac{32 \times 60}{15 \times 12} = \underline{10{\cdot}7.}$$

Measurement of Oxygen Consumption; the 'Direct Method' of Warburg.

The respiration of most living cells, as opposed to many enzyme preparations, results in the consumption of oxygen and the evolution of carbon dioxide. Where oxygen and carbon dioxide are the only gases involved, oxygen consumption can be measured by absorbing the CO_2 in alkali placed in the centre well. This keeps the CO_2 pressure zero in the gas phase and the net gaseous exchange is then due to the oxygen consumed. This is the basis of the 'Direct Method' of Warburg for determining oxygen uptake.

One of the major disadvantages of the method lies in the fact that the atmosphere must be free from CO_2, and it has been shown for certain tissues (brain, liver, etc.) that oxygen uptake is stimulated by the presence of CO_2. Consequently, results obtained by the 'Direct Method' may not be a true reflection of metabolism under normal conditions. This difficulty may be circumvented by using Warburg's 'Indirect Method', details of which may be found in the recommended books.

The 'Direct Method' is used in two main ways, for the determination of (*a*) the rate of oxidation of a substrate, i.e. the Q_{O_2} value, and (*b*) the total oxygen consumption in the presence of a measured quantity of substrate. It is customary to determine both in the same experiment.

In determinations of both rate and total amount of oxygen uptake the status of the endogenous respiration (i.e. respiration of the tissue in the absence of external substrate) must be assessed and often leads to uncertainty. This uncertainty arises because the presence of the added substrate may or may not suppress the endogenous respiration, and the problem presented is whether a constant rate of endogenous respiration should be assumed and the values deducted from the oxygen uptake observed in the presence of the substrate. It is customary in published work to report the endogenous respiration and to state whether or not it has been

subtracted from the values recorded for the oxidation of the substrate.

Oxidative Assimilation

When measurements of total oxygen consumption were made with non-proliferating suspensions of certain micro-organisms metabolizing various substrates it was discovered that oxidation ceased at levels corresponding to 30-60 per cent. oxidation of the substrate. Analysis showed that no substrate remained in the manometer flask and addition of a further quantity of substrate permitted resumption of oxidation. This led to the concept of *oxidative assimilation* and it has been demonstrated, mainly by Clifton and his colleagues, that energy derived from oxidation of certain substrates is utilized by the micro-organisms to enable a portion of the substrate to be synthesized into cellular components, as indicated by an increase in the carbon content of the cells. Thus where total oxygen uptake corresponds to 40 per cent. oxidation the remaining 60 per cent. has been assimilated by the cells.

Example 8.4.—Oxidation of ethanol by the colourless alga *Prototheca zopfii* was investigated manometrically and 5 μmoles of ethanol found to give a total oxygen consumption of 187 μl. Over the same period the endogenous respiration was 36 μl. What was the percentage of ethanol synthesized into cellular material?

Assuming no suppression of endogenous respiration, the true oxygen uptake is $187 - 36 = 151$ μl.

$$= \frac{151}{22{\cdot}4} = 6{\cdot}74 \text{ } \mu\text{moles.}$$

For complete oxidation of ethanol to carbon dioxide and water

$$C_2H_5OH + 3O_2 = 2CO_2 + 3H_2O$$

3 moles of oxygen are required per mole of ethanol.

Thus 5 μmoles ethanol require 15 μmoles O_2 for complete oxidation.

$$\text{Percentage oxidation} = \frac{6{\cdot}74}{15} \times 100 = 44{\cdot}9.$$

Hence percentage synthesized into cellular material = 55·1.

Carbon Dioxide Production by the 'Direct Method.'

Subject to the possible errors already mentioned, one is able to determine carbon dioxide evolution by the 'Direct Method' and hence obtain the R.Q. Two flasks are set up with the same contents and carbon dioxide is absorbed by alkali in one but not in the other. The former gives the oxygen uptake, whereas the

gas exchange in the latter is due to the combined effect of oxygen consumption and carbon dioxide evolution; the carbon dioxide output can therefore be determined. Flask 1 contains alkali. Let h_{O_2} be the observed manometer change, x_{O_2} the amount of oxygen absorbed and $k_{1_{O_2}}$ the flask constant for oxygen. Flask 2 has no alkali. Let h be the observed manometer change, h_{CO_2} the movement due to the carbon dioxide, x_{CO_2} the amount of carbon dioxide evolved and $k_{2_{O_2}}$ and $k_{2_{CO_2}}$ the flask constants for oxygen and carbon dioxide respectively.

Now, the amount of oxygen absorbed is obtained from flask 1 and is given by

$$x_{O_2} = h_{O_2} k_{1_{O_2}}$$

For flask 2 the observed manometer change is

$$h = h_{O_2} + h_{CO_2}$$

$$= \frac{x_{O_2}}{k_{2_{O_2}}} + \frac{x_{CO_2}}{k_{2_{CO_2}}}$$

Whence

$$x_{CO_2} = \left(h - \frac{x_{O_2}}{k_{2_{O_2}}}\right) k_{2_{CO_2}} \quad . \quad . \quad . \quad (8.3)$$

Thus, obtaining x_{O_2} from flask 1, x_{CO_2} may be obtained from the observed manometer change in flask 2.

Example 8.5.—Equal weights of rat liver slices were placed in two Warburg flasks together with saline. Alkali was added to the centre well of one flask which had an oxygen constant of 1·55. The other flask had carbon dioxide and oxygen constants of 1·77 and 1·42 respectively. After 60 minutes the alkali-containing flask showed a manometer change of 105 mm. and the other manometer recorded a fall in level of 45·5 mm. Determine the respiratory quotient of the tissue slices.

The oxygen absorbed is given by the alkali-containing flask

$$x_{O_2} = -1{\cdot}55 \times 105 = -162{\cdot}8 \ \mu l.$$

The carbon dioxide evolved is given by the expression

$$x_{CO_2} = \left[-45{\cdot}5 - \left(\frac{-162{\cdot}8}{1{\cdot}42}\right)\right] 1{\cdot}77$$

$$= [-45{\cdot}5 + 114{\cdot}7]\ 1{\cdot}77$$

$$= +122{\cdot}5 \ \mu l.$$

Hence R.Q.

$$= \frac{122{\cdot}5}{162{\cdot}8} = \underline{0{\cdot}75.}$$

Note that this example represents an ideal case where the weights of tissue are equal in the two flasks. In actual practice this is very

difficult to achieve and therefore results must be reduced to comparable values by dividing the observed manometer changes by the weight of tissue present in the flasks. Then h_{O_2} and h are in terms of mm. per mg., x_{O_2} in terms of μl. per mg. and substitution of these values in equation 8.3 gives x_{CO_2} in terms of μl. per mg. The R.Q. is then obtained from the x_{CO_2}/x_{O_2} ratio.

Retention of Carbon Dioxide by Buffers

At physiological pH values carbon dioxide reacts with buffer solutions and is converted into bicarbonate. This means that in the presence of buffers the observed manometer change will not include all the CO_2 evolved and the value for x_{CO_2} will be decreased. Phosphates and proteins have this effect and cause retention of CO_2.

$$Na_2HPO_4 + CO_2 + H_2O = NaH_2PO_4 + NaHCO_3.$$

Consequently manometric determinations employing phosphate buffer or serum as media are subject to error and the retained CO_2 must be liberated from the bicarbonate at the end of the experiment in order to obtain the total CO_2 evolution. Addition of acid from a side bulb of the flask liberates the CO_2, but since the original medium and tissue probably contained a small quantity of CO_2 initially, a third manometer must be set up in which acid is added at the beginning of the experiment. This gives the bound CO_2 initially present and must be subtracted from the amount given by the second manometer to obtain the true amount of CO_2 evolved during the experiment. Consequently for experiments using phosphate buffer solutions three manometers are necessary, one with alkali to record oxygen uptake and two without alkali, one of which has acid added at the beginning and the other at the end of the experiment, in order to determine the CO_2 evolved.

Carbon Dioxide Production by Other Methods.

The 'Direct Method' is not widely used for CO_2 output determinations and is replaced either by the 'Indirect Method' of Warburg or the First or Second Methods of Dickens and Šimer or by the method of Dixon and Keilin. Discussion of these methods lies outside the scope of a book of this size and the student is referred to the prescribed texts for details.

The Use of Bicarbonate-Carbon Dioxide Buffers.

In manometry bicarbonate-CO_2 buffers find great application and the relationship between the bicarbonate and CO_2 concentrations and the pH is of considerable importance.

When CO_2 dissolves in aqueous solution, the following equilibria may be envisaged:

$$CO_2(\text{gas}) \rightleftharpoons CO_2(\text{dissolved in solution}) \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3'.$$

The dissociation constant for the first dissociation of carbonic acid is given by the expression

$$K_{a1} = \frac{(H^+)(HCO_3')}{(H_2CO_3)} \quad . \quad . \quad . \quad (8.4)$$

but since (H_2CO_3) is dependent on the concentration of dissolved CO_2 (which in turn is governed by the partial pressure of CO_2 in the gas phase) equation 8.4 may be rewritten as

$$K_{a1} = \frac{(H^+)(HCO_3')}{(CO_2)} \quad . \quad . \quad . \quad (8.4a)$$

where (CO_2) is the concentration of dissolved gas. The Henderson–Hasselbalch equation (2.10) enables the following relationship to be written:

$$pH = pK_{a1} + \log \frac{(HCO_3')}{(CO_2)} \quad . \quad . \quad (8.5)$$

As discussed in Chapter II, the activity of the bicarbonate ion must be used for strict accuracy. However, to make for ease in calculation, the activity coefficient of the bicarbonate ion may be included in the pK_a term when the concentrations of HCO_3' and CO_2 can then be used. Thus

$$pH = pK_{a1}' + \log \frac{[HCO_3']}{[CO_2]} \quad . \quad . \quad (8.6)$$

where

$$pK_{a1}' = pK_{a1} + \log f_{HCO_3'} \quad . \quad . \quad (8.7)$$

pK_{a1}' has the value of 6·317 at infinite dilution and 38°. The value is corrected for concentrations other than infinite dilution by the expression

$$6{\cdot}317 - 0{\cdot}5\sqrt{I} \quad . \quad . \quad . \quad (8.8)$$

where I is the ionic strength. Manometric experiments rarely employ bicarbonate concentrations higher than 0·1 M and it has been shown that the correction for this concentration is small enough to be neglected for most purposes Although equation 8.6 does not take into account the second dissociation constant of carbonic acid, its effect may be ignored provided the pH is less than 8.

A problem arises in connexion with the value taken for the bicarbonate concentration, for it represents the contribution of both the added bicarbonate and the bicarbonate arising from the dissociation of carbonic acid. However, certain working rules have been established, and providing the hydrogen ion concentration is one-hundredth of the $NaHCO_3$ concentration, the bicarbonate concentration for use in equation 8.6 may be taken as equal to the concentration of $NaHCO_3$ added. The error involved in neglecting the carbonic acid contribution is less than 0·1 per cent. For example, at pH 6, $[H^+] = 10^{-6}$ M and the lowest bicarbonate concentration permissible would be 10^{-4} M; at pH 7 the bicarbonate concentration should be greater than 10^{-5} M. There is, therefore, over the physiological range of pH, no practical difficulty in providing sufficient bicarbonate to eliminate errors due to neglect of the second dissociation constant of carbonic acid. For many purposes it is even possible to cut the above relationship by one-tenth without introducing large errors, e.g. providing the bicarbonate concentration is ten times the hydrogen ion concentration, $[HCO_3']$ may be taken as equal to the added bicarbonate concentration.

The CO_2 concentration in equation 8.6 is in terms of moles per litre of solution whereas the value usually known is the percentage or partial pressure in mm. of mercury. Conversion of these units to the former is effected as follows.

If $(CO_2\%)$ is the percentage CO_2 at atmospheric pressure P, and α is the solubility of CO_2 (in ml. gas per ml. liquid), then the partial pressure of CO_2 will be

$$p_{CO_2} = \frac{P(CO_2\%)}{100},$$

and under standard conditions (760 mm.) $p_{CO_2} = \frac{P(CO_2\%)}{760 \times 100}$ atmospheres. The quantity of CO_2 (in ml.) dissolved per litre

of solution will be p_{CO_2} multiplied by 1000α, and to convert this to moles per litre it must be divided by 22400. Hence

$$[CO_2] \text{ in moles/litre} = \frac{P(CO_2\%)1000\alpha}{760 \times 100 \times 22400} \quad . \quad (8.9)$$

The Henderson–Hasselbalch equation then becomes

$$pH = pK_{a_1}' + \log [HCO_3'] - \log P(CO_2\%) - \log \frac{\alpha}{760 \times 2240} \quad . \quad . \quad . \quad . \quad (8.10)$$

$$\text{or } pH = pK_{a_1}' + \log [HCO_3'] - \log p_{CO_2} - \log \frac{\alpha}{760 \times 22{\cdot}4} \quad . \quad . \quad . \quad . \quad (8.11)$$

Inspection of this equation, after substituting the value of 6·317 for pK_{a_1}' at 38°, reveals the fact that it is impossible to measure CO_2 at pH 7 unless there is CO_2 in the atmosphere, for as $[CO_2]$ tends to zero, the $[HCO_3']/[CO_2]$ ratio increases and hence the pH also increases. Under normal conditions the concentration of CO_2 in the atmosphere is very small and in order to maintain a physiological pH value of 7 it is necessary either to decrease appreciably the bicarbonate concentration or to supply CO_2 in the atmosphere of the manometer flask. The former procedure is impracticable because, as already mentioned, retention of CO_2 by tissue buffers increases the bicarbonate concentration, and hence addition of CO_2 to the gas phase is the only practicable method. By taking a fixed bicarbonate concentration and varying the partial pressure of CO_2, or vice versa, it is possible to adjust the pH to any value in the physiological range of 6 to 8. Bicarbonate ion cannot exist in solution at pH 5 or below, therefore all CO_2 liberated remains in the gas phase under these conditions.

Example 8.6.—Bicarbonate–Ringer solution contains 0·025 M bicarbonate and is in equilibrium with an atmosphere containing 5 per cent. CO_2 at 38°. The solubility coefficient of CO_2 is 0·537 and pK_{a_1}' is 6·317 at 38°. What is the initial pH value of the solution? Atmospheric pressure is 740 mm.

$$\begin{aligned} pH &= pK_{a_1}' + \log [HCO_3'] - \log \frac{P(CO_2\%)\alpha}{760 \times 2240} \\ &= 6{\cdot}317 + \log 0{\cdot}025 - \log \frac{740 \times 5 \times 0{\cdot}537}{760 \times 2240} \\ &= 6{\cdot}317 + (\bar{2}{\cdot}3979) - (\bar{3}{\cdot}0672) \\ &= 6{\cdot}317 - 1{\cdot}6021 + 2{\cdot}9328 \\ &= 7{\cdot}65. \end{aligned}$$

SUGGESTED READING

DIXON, M. (1951). *Manometric Methods*, 3rd ed. Cambridge University Press.

UMBREIT, W. W., BURRIS, R. H., & STAUFFER, J. F. (1949). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, 2nd ed. Minneapolis: Burgess Publishing Co.

PROBLEMS

8.1. The following data were obtained in experiments to calibrate five Warburg manometers by the mercury method. The volumes (in ml.) of the flasks to the zero point of the manometers were respectively:

(*a*) 21·24; (*b*) 21·00; (*c*) 19·22; (*d*) 22·21; (*e*) 20·54

The solubility of oxygen at 37° is 0·0239 and the density of the manometer fluid 1·030.

Determine the oxygen constants of these manometers at 37° for 3 ml. fluid content of the flasks.

8.2. The carbon dioxide constants of four Warburg manometers were determined by means of the bicarbonate method.

2 ml. of a solution containing 0·3361 g. sodium bicarbonate per litre were pipetted into the main compartments and 1 ml. of 2 N sulphuric acid was placed in the side bulbs. When the reaction had ceased, the observed manometer changes (in mm.) were (*a*) +93; (*b*) +102·5; (*c*) +88; (*d*) +98·5 and the thermobarometer showed a difference of +3·0 mm.

Determine the constants.

8.3. Oxygen constants for Warburg manometers may be obtained by measuring the nitrogen evolved when excess of alkaline hydrazine solution is allowed to react with potassium iodate. The nitrogen constant thus obtained is practically identical with the oxygen constant and may be used as such. The reaction involved is:

$$2KIO_3 + 3N_2H_4 = 2NaI + 3N_2 + 6H_2O.$$

1 ml. of a solution containing 0·3210 g. potassium iodate per 250 ml. was pipetted into the side bulbs of a series of manometers and 2 ml. of an alkaline hydrazine solution were placed in the main compartment. After the reaction had taken place the following manometer changes (in mm.) were recorded: (*a*) +128; (*b*) +109; (*c*) +136; (*d*) +145, and the thermobarometer had changed by −2 mm.

What are the constants for these manometers?

8.4. Calculate the respiratory quotients for the complete oxidation of the following compounds: (*a*) glycerol, (*b*) pyruvic acid, (*c*) oxaloacetic acid, (*d*) α-ketoglutaric acid, (*e*) citric acid, (*f*) glutamic acid, (*g*) lysine, (*h*) capric acid, (*j*) stearic acid.

8.5. Air freed from moisture and carbon dioxide contains 21·00 per cent. oxygen and 78·06 per cent. nitrogen by volume. What are the partial pressures of these gases when the atmospheric pressure is 76 cm. Hg?

8.6. Using a gas phase containing 5 per cent. carbon dioxide, what concentration of bicarbonate must be employed in order to obtain media of pH values 6·0, 6·5, 7·0 and 7·5? At 38°, pK_{a_1}' for carbonic acid is 6·317 and α_{CO_2} is 0·537. Assume atmospheric pressure to be 760 mm.

8·7. The following rates of evolution of carbon dioxide, produced by the action of a preparation of a decarboxylase on L (+) ornithine, were obtained using a Warburg respirometer:

μl. CO_2 evolved per 5 minutes	*Ornithine concentration*, mM.
10·0	0·556
36·4	3·33
52·7	8·34
66·6	25·0

Determine by an accurate graphical method the maximum rate of evolution attainable under these conditions, and derive any relationship you employ.

(Leeds Honours Course Finals, 1951.)

8.8. A solution of oxaloacetic acid of unknown concentration was analysed manometrically by the aniline-citrate method which catalyses the decarboxylation of the keto acid. After deduction of the appropriate control the carbon dioxide evolution from 1 ml. of the solution was 168 μl.

Determine the molarity of the oxaloacetic acid solution and also express the concentration in terms of percentage (w/v).

8.9. Briefly outline how you would determine *three* of the following: (*a*) L-histidine; (*b*) pyruvic acid; (*c*) succinic acid; (*d*) hydrogen peroxide, using manometric methods, including in your answer the exact constituents of the media used in the flasks; details of operation of the manometers are not required.

Urea can be determined manometrically using a urease preparation which catalyses the reaction:

$$CO(NH_2)_2 + H_2O = CO_2 + 2NH_3.$$

Using 1 ml. of a urea solution and a buffer of pH 5, the gas evolutions recorded on control and reaction manometers were respectively 8·7 and 83·3 μl. (N.T.P.). Calculate the molarity of the urea solution employed.

(Leeds Honours Course Finals, 1949.)

8.10. The oxidation of glycerol by the alga *Prototheca zopfii* has been investigated by Barker and the effect of glycerol concentration on total oxygen consumption is recorded below:

Glycerol present μmoles	*Total oxygen consumed* (μl.)	
	Control (no substrate)	*Observed with substrate (no correction applied)*
6·90	53	170
13·8	75	329
27·6	106	659
55·2	161	1292

Determine the extent to which oxidative assimilation has occurred and ascertain whether these data are consistent with the supposition that an increasing substrate concentration suppresses endogenous respiration of the alga.

(After BARKER (1936), *J. cell. comp. Physiol.*, **8**, 231.)

8.11. The effect of variation of bacterial concentration on the oxidation of glucose by *Pseudomonas aeruginosa* was studied and the following results obtained:

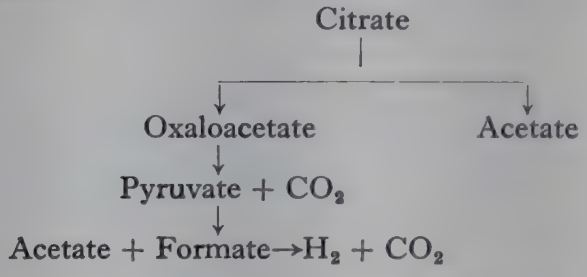
Relative concentration of bacteria	*Oxygen consumption* (μl.)	
	Endogenous	5 μmoles *glucose*
1	45	491
2	95	540
4	200	645

Endogenous respiration has not been subtracted from the observed uptake in the presence of substrate.

Is there any evidence for oxidative assimilation by these cells and, if so, to what extent? What conclusions may be drawn about the effect of the substrate on the endogenous respiration of the organism?

(Adapted from NORRIS, CAMPBELL & NEY (1949), *Canad. J. Res.*, C, **27**, 157.)

8.12. *Aerobacter aerogenes* ferments citric acid in accordance with the following scheme:



Arsenite, at a concentration of $2{\cdot}5 \times 10^{-3}$ M, inhibits the fermentation, and to investigate its locus of action manometers were set up containing 2·5 μmoles of citrate with and without arsenite. Total gas evolution was measured in each case in an atmosphere of nitrogen and 5 per cent. carbon dioxide, and after deduction of the respective controls, values of 48μl. and 139 μl. respectively were obtained in the presence and absence of arsenite. From these data deduce the probable point of action of arsenite on the fermentation. What further experiment would you carry out to confirm your conclusions?

(After DAGLEY & DAWES (1953), *J. Bact.*, **66**, 259.)

8.13. The effect of sodium azide and 2:4 dinitrophenol (DNP) on the aerobic utilization of glucose by a strain of baker's yeast has been investigated.

Each Warburg vessel contained 0·1 ml. of 0·1 M glucose, 0·1 ml. of NaN_3, DNP or water and 1·8 ml. of a 0·66 M phosphate buffer (pH 6·0) suspension of yeast. Results obtained were as follows:

Reagent	*Oxygen consumed* μl.	*CO_2 evolved* μl.
—	466	481
10^{-3} M DNP	495	779
10^{-4} M NaN_3	543	691
5×10^{-4} M NaN_3	104	520

Determine the respiratory quotient in the presence and absence of these reagents. What explanation can you offer for the observed results?

(After PICKETT & CLIFTON (1941), *Proc. Soc. exp. Biol. Med.*, **46**, 443.)

8.14. The metabolism of glucose by rat liver slices was investigated manometrically. Equal weights of slices were placed in three Warburg flasks containing phosphate buffer of pH 7·0 and alkali was added to the centre well of one of these. 5 N sulphuric acid was added to the side arms of the other two flasks which were used to determine carbon dioxide evolution. At the start of the experiment acid was tipped in one of the flasks which had a carbon dioxide constant of 1·85 and the manometer change was +21 mm.

At the end of the experiment the alkali-containing flask recorded −121 mm. pressure (k_{O_2}, 1·65 for this manometer) and the other manometer, after tipping the acid, gave a reading of −8·0 mm. The constants for this latter flask were k_{O_2}, 1·59 and k_{CO_2}, 1·80.

Determine the oxygen consumption and carbon dioxide evolution and hence the respiratory quotient.

8.15. The effect of aeration on the endogenous and glucose metabolism of non-proliferating suspensions of *Sarcina lutea* has been investigated. The cells, suspended in water, were incubated at 37° and a vigorous stream of sterile air passed through the culture. At intervals samples of cells were withdrawn, centrifuged, washed and set up in Warburg manometers to determine the oxygen consumption in the presence and absence of 5 μmoles of glucose. The following data refer to manometer readings at 60 minutes, obtained with 1 ml. bacterial suspension per manometer :

Period of aeration hours	*No substrate*		5 μmoles *glucose*		*Bacterial dry weight* mg./ml.
	Manometer reading mm.	*Flask constant for* O_2	*Manometer reading* mm.	*Flask constant for* O_2	
0	−272	1·64	−329	1·69	31·0
1	−158	1·47	−245	1·55	31·2
2	−70	1·57	−183	1·67	32·3
3·5	−34·5	1·56	−191	1·60	32·6
4·5	−14·0	1·43	−213	1·52	33·5

Determine the Q_{O_2} values for endogenous and glucose metabolism and comment on the effect of aeration on these quotients.

(After Dawes & Holms, unpublished data.)

CHAPTER IX

BACTERIAL GROWTH

The Bacterial Growth Cycle.

If a number of living bacteria are inoculated into a suitable nutrient medium their subsequent growth cycle may be conveniently divided into three well-defined phases:

(*a*) the lag phase, before cell division commences,

(*b*) the logarithmic growth phase, when the cells are dividing at constant rate, and

(*c*) the stationary phase, when cell division ceases.

These are shown in Fig. 9.1. Other phases may be distinguished, such as the acceleration phase between (*a*) and (*b*) and the deceleration phase between (*b*) and (*c*), but quite often the transition from lag to logarithmic and from logarithmic to stationary phase is so sharp that these other phases may be non-existent.

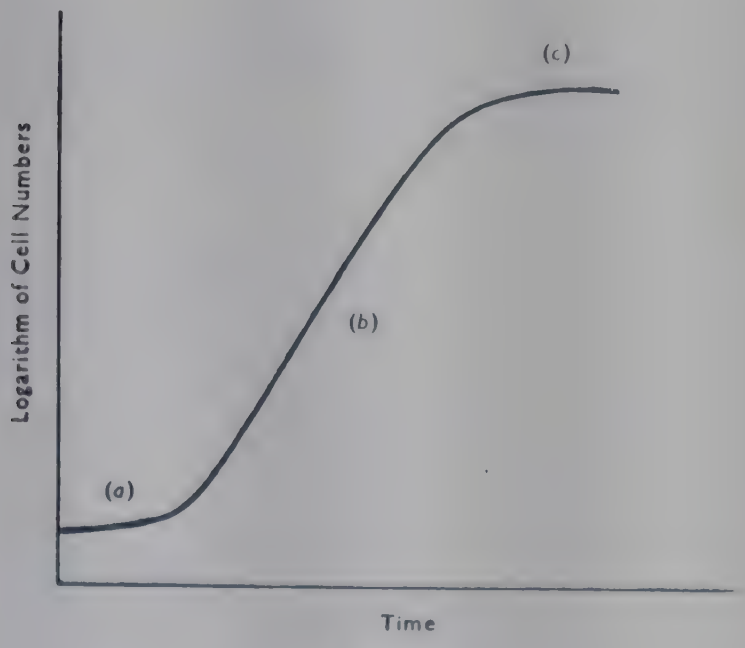


FIG. 9.1

Typical bacterial growth cycle.

The Logarithmic Growth Phase.

If the case of an individual organism in the medium is considered it will be found to increase in size until, finally, division into two daughter cells occurs, each of which in turn increases in size and divides. The period between one cell division and the next is called the 'generation time', and when observations are made on a single cell this value is found to fluctuate quite considerably. However, in most bacterial cultures we are dealing with populations of many millions of cells and under these circumstances the average generation time for all cells in the culture is fairly constant, and is referred to as the *mean generation time*.

If n_0 cells are inoculated into a nutrient medium, then at the end of one generation time there will be $2n_0$ cells, at the end of two generations $4n_0$ cells (or 2^2n_0) and at the end of x generations 2^xn_0 cells. If n cells are present at the end of x generations, then

$$n = n_0 \cdot 2^x \quad . \quad . \quad . \quad (9.1)$$

Taking logarithms, $\log n = \log n_0 + x \log 2$

and

$$\frac{\log n - \log n_0}{\log 2} = x.$$

Let T be the mean generation time and t the time taken for the population to increase logarithmically from n_0 to n, then

$$\frac{t}{T} = x$$

and substituting in the above equation

$$\frac{\log n - \log n_0}{t} = \frac{\log 2}{T} \quad . \quad . \quad . \quad (9.2)$$

When bacterial growth is followed experimentally, results are usually expressed by a graph in which the logarithm of the cell count is plotted against the time. For the logarithmic phase of growth this results in a linear plot for log n against time. From such graphical representation the above derived relationship (equation 9.2) follows by similar triangles (Fig. 9.2).

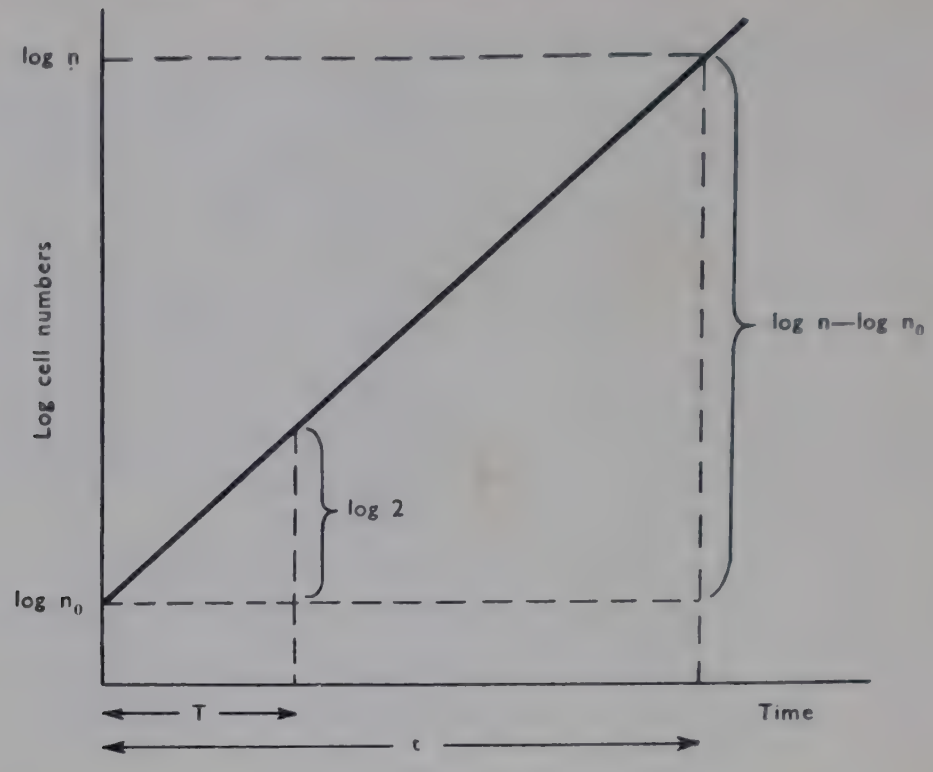


FIG. 9.2

Logarithmic plot of bacterial cell numbers against time.

An alternative method of recording growth rates is by means of the growth constant k. The rate of increase of a bacterial population is given by the expression

$$\frac{dn}{dt} = kn$$

and integration of this yields

$$\ln \frac{n}{n_0} = kt \quad . \quad . \quad . \quad . \quad (9.3)$$

where the constant k is known as the *growth constant*. Therefore the slope of the line in the plot of $\log n$ against t (Fig. 9.2) is $k/2{\cdot}303$. With k evaluated, the mean generation time is obtained from the equation

$$\ln 2 = kT$$

whence

$$T = \frac{0{\cdot}693}{k} \quad . \quad . \quad . \quad (9.4)$$

Example 9.1.—An inoculum of 4×10^5 bacteria grew in a medium without lag to a population of $3{\cdot}68 \times 10^7$ cells in 6 hours and had not then reached the stationary phase. What was the mean generation time of the organism in this medium?

Data given: $n_0 = 4 \times 10^5$ and $n = 3{\cdot}68 \times 10^7$. $t = 360$ minutes.

Where T is the mean generation time, since there was no lag period:

$$\frac{\log n - \log n_0}{t} = \frac{\log 2}{T}$$

$$\frac{7{\cdot}5658 - 5{\cdot}6021}{360} = \frac{0{\cdot}3010}{T}$$

$$T = \frac{0{\cdot}3010 \times 360}{1{\cdot}9637} = 55{\cdot}2 \approx \underline{55 \text{ minutes}}.$$

Example 9.2.—25 ml. of peptone medium were inoculated with 4×10^6 cells of *Escherichia coli* and incubated at 37° C. The stationary phase of 3×10^9 cells per ml. was reached after 284 minutes and no lag phase occurred. What was the mean generation time in the peptone medium?

Data given: $n_0 = 4 \times 10^6$ cells per 25 ml., $n = 3 \times 10^9$ cells per ml. and $t = 284$ minutes.

Note particularly that n_0 and n are not expressed in the same units, and therefore n_0 must be converted to cells per ml. before the calculation can be carried out.

$$n_0 = \frac{4 \times 10^6}{25} = 1{\cdot}6 \times 10^5 \text{ cells per ml.}$$

$$T = \frac{284 \times 0{\cdot}3010}{9{\cdot}4771 - 5{\cdot}2041} = \frac{85{\cdot}48}{4{\cdot}2730} = \underline{20 \text{ minutes}}.$$

The Lag Phase.

The examples cited are ones where the inoculum of n_0 cells commences to divide immediately without a lag phase. If, as frequently happens, there is a period of lag before logarithmic growth begins, the time t measured for the increase in population to n is not occupied exclusively by cell division but includes also the time L—the length of the lag phase (Fig. 9.3).

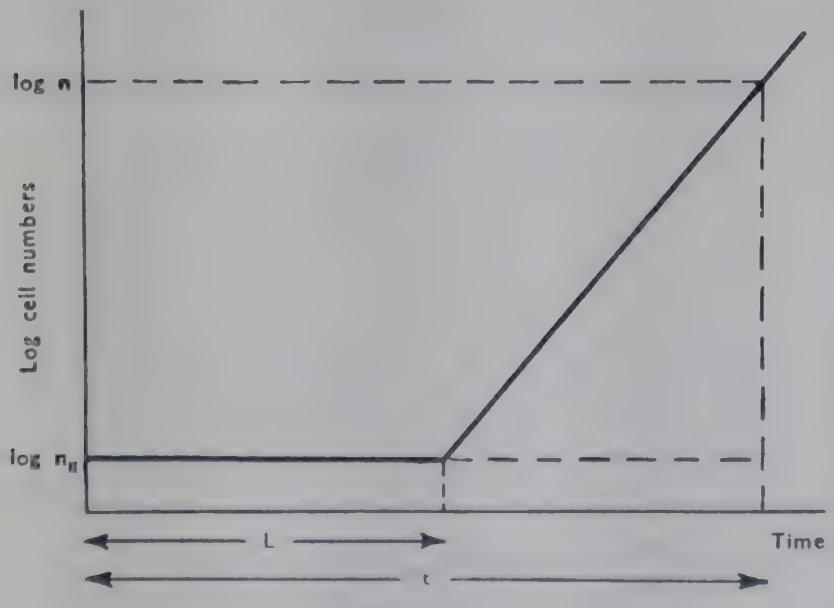


FIG. 9.3

Lag and logarithmic phases of growth cycle.

L

In this connexion one point remains to be made. The transition from lag to logarithmic phase is not always sharp, and difficulty may be experienced in deciding exactly where the lag phase ends. To overcome this difficulty, the length of the lag phase is conventionally defined as the intercept on the line drawn through the logarithm of the inoculum population, parallel to the time axis, obtained by extrapolating the growth curve backwards. This is made clear in Fig. 9.4.

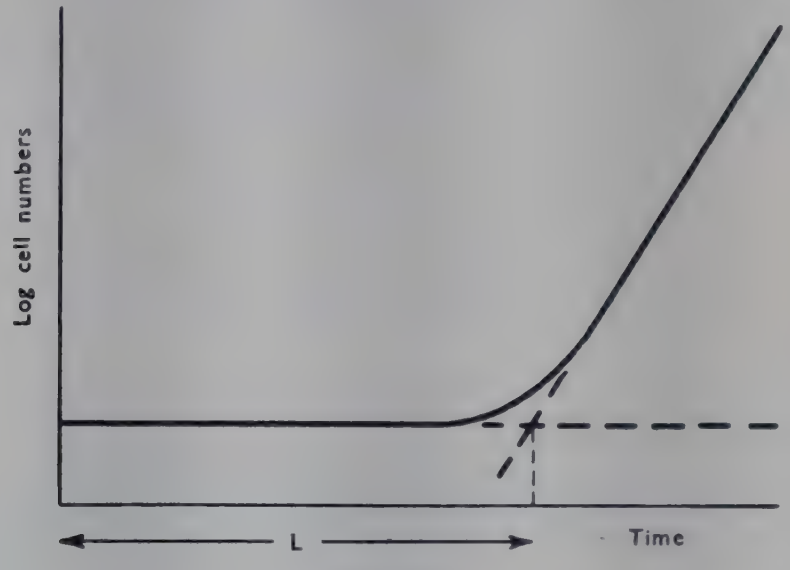


FIG. 9.4
Graphical determination of the length of the lag phase.

Thus, if we know the inoculum population, n_0, the population n after time t, and the mean generation time of the cells T, it is possible to decide whether or not a lag phase occurred, since

$$\frac{\log n - \log n_0}{t - L} = \frac{\log 2}{T} \quad . \quad . \quad . \quad (9.5)$$

of which L is the only unknown.

Example 9.3.—A glucose-ammonium salt medium was inoculated with 5×10^5 cells of *Escherichia coli*. After 300 minutes the culture was still in the logarithmic phase of growth with a population of 35×10^6 cells. If the mean generation time of the organism in this medium is 40 minutes, determine whether or not a lag phase was manifest and, if so, its duration.

Data given: $n_0 = 5 \times 10^5$, $n = 35 \times 10^6$ (after 300 minutes) and $T = 40$ minutes.

These data enable the time taken for n_0 cells to increase to n to be calculated. Thus

$$\frac{\log n - \log n_0}{t} = \frac{\log 2}{T}$$

Substituting the known values

$$\frac{7{\cdot}5441 - 5{\cdot}6990}{t} = \frac{0{\cdot}3010}{40}.$$

Therefore $$t = \frac{1{\cdot}8451 \times 40}{0{\cdot}3010} = \frac{73{\cdot}804}{0{\cdot}3010} = 245 \text{ minutes.}$$

Thus the time taken for actual cell division to give rise to a population of 35×10^6 cells was 245 minutes, whereas the observed time was 300 minutes. Hence the lag period $300 - 245 = 55$ minutes occurred.

Total Growth.

The total growth of bacteria obtained in a given medium is determined by various factors. These are:

(*a*) exhaustion of an essential nutrient,
(*b*) development of an adverse pH value, and
(*c*) accumulation of end products of metabolism which, taken in conjunction with the pH value of the medium, may become toxic to the organism and prevent further growth.

Provided that factors (*b*) and (*c*) are not limiting growth and that concentrations of all potentially growth limiting nutrient substances are adjusted so that they are in excess compared to one given nutrient, then a linear relationship exists between the total growth (i.e. stationary population) and the concentration of that latter substance. The graph of such a relationship is shown in Fig. 9.5, and where linearity breaks down, factors other than nutrient concentration are limiting growth.

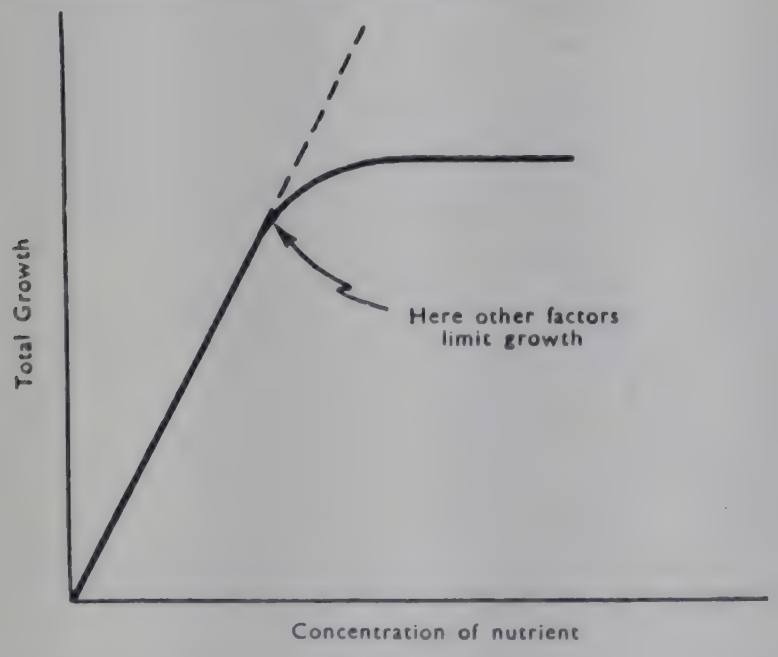


FIG. 9.5
Relationship between total bacterial growth and concentration of a given nutrient.

Example 9.4.—The relationship between the total growth of *Aerobacter aerogenes* and the concentration of citric acid in unaerated citrate-ammonium salt medium is linear up to a citrate concentration of 6×10^{-2} M, at which value a population of 950×10^6 bacteria per ml. is supported. Higher concentrations of citrate produce no increase in crop.

50 ml. of a medium containing an unknown amount of citric acid were inoculated with *A. aerogenes* and the unaerated stationary population measured. If this final population was 725×10^6 bacteria per ml., how many milligrams of citric acid were present in the medium?

The molecular weight of citric acid is 189.

Therefore 6×10^{-2} M citric acid

$= 189 \times 6 \times 10^{-2}$ grams per litre

$= 11{\cdot}34$ g. per litre

$= 0{\cdot}567$ g. per 50 ml.

Thus 950×10^6 cells are supported by 0·567 g. per 50 ml. and 725×10^6 cells are supported by $\frac{0{\cdot}567 \times 725}{950} = 0{\cdot}433$ g. per 50 ml.

$= \underline{433 \text{ mg. per } 50 \text{ ml.}}$

Diauxic Growth Cycles.

In certain circumstances more complex growth cycles may be encountered, the most familiar being two consecutive logarithmic growth phases separated by a lag phase. This phenomenon of 'diauxie', i.e. double growth, is observed when certain bacteria are grown in media containing limiting amounts of two different carbohydrates. The first growth phase corresponds to the exclusive utilization of one of the sugars followed by a period of adaptation before the other substrate is metabolized and growth

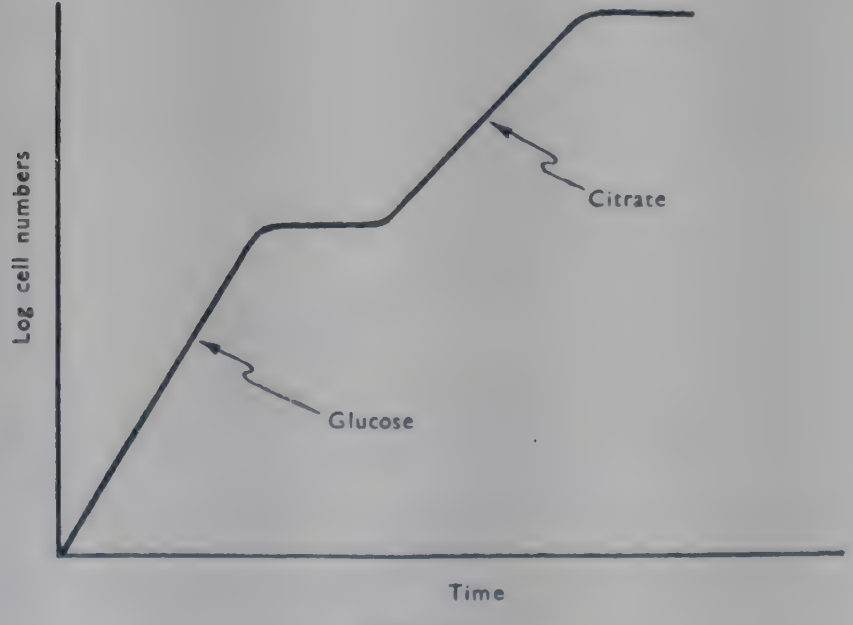


FIG. 9.6

Diauxic growth curve of *Aerobacter aerogenes* in citrate medium containing a limiting concentration of glucose.

recommences. A typical diauxic growth curve is shown in Fig. 9.6, where the substrates are glucose and citric acid respectively.

SUGGESTED READING

HINSHELWOOD, C. N. (1946). *Chemical Kinetics of the Bacterial Cell*, Chaps. II, III. Oxford University Press.

MONOD, J. (1949). The growth of bacterial cultures. *Ann. Rev. Microbiol.*, **3**, 371.

PROBLEMS

9.1. *Aerobacter aerogenes* was inoculated simultaneously into three different media: peptone and two chemically defined media containing respectively glucose and citric acid as the sole sources of carbon. The inoculum in each case was 3×10^5 cells per ml. After 4·5 hours measurements of the cell population in each culture revealed values of 9732×10^6, $41{\cdot}11 \times 10^6$ and $1{\cdot}427 \times 10^6$ cells per ml. for peptone, glucose and citrate respectively. The citrate culture was the only one to display a lag period, the duration of which was 90 minutes, and the cells in each culture were still growing logarithmically.

What were the mean generation times for the organism in these media? Comment on the results.

9.2. The following data were obtained in an experiment in which anaerobically grown *Aerobacter aerogenes* was inoculated into a glucose-ammonium sulphate medium and grown with gentle aeration at 37° C. The inoculum used was $2{\cdot}0 \times 10^6$ bacteria per ml.

Time (minutes)	150	200	250	280	310	340	370	400
Bacterial population (millions per ml.)	14·1	38·9	104·7	190·6	346·7	616·5	794·2	812·7

Determine graphically the mean generation time of the organism under these conditions, whether or not a lag period occurs and, if so, its duration.

9.3. A glucose-ammonium salt medium was inoculated with 2·5 million cells per ml. of *Aerobacter aerogenes* and grown initially without aeration. After a period, a gentle stream of air was passed through the culture until growth ceased. From the protocol given below, determine the mean generation time of the organism for unaerated and aerated growth and also the time after inoculation that the air stream was turned on.

Time (minutes)	*Bacterial population* (millions per ml.)
100	13·80
130	23·44
160	39·81
190	66·07
220	117·5
250	223·9
280	436·5
310	812·8
340	1122·0
370	1148·0

9.4. *Escherichia coli* was grown in a medium containing 0·5 g. per litre fructose as the sole carbon source, the sugar being completely utilized within 538 minutes. The inoculum population used was 5×10^4 bacteria per ml. If the relationship between stationary population and fructose concentration is linear up to 0·80 g. per litre sugar, at which concentration the population supported is $3{\cdot}2 \times 10^8$ bacteria per ml., determine the mean generation time of the organism in this medium. What length of time would elapse before growth ceased in the presence of excess fructose if the same size inoculum were used? It may be assumed that the breakdown in linearity of the total growth and fructose concentration relationship is quite sharp.

9.5. The addition of certain compounds to cultures of *Aerobacter aerogenes* which are displaying very long lag periods in glucose-ammonium sulphate media has the effect of either shortening or prolonging the length of this lag phase. In an experiment to determine the effect of various organic acids, all added to a concentration of 0·06 g. per litre, each tube received an inoculum of 2×10^4 bacteria per ml. Comparison with a control culture containing no added organic acid revealed the fact that all the cultures were growing at the same rate with a mean generation time of 40 minutes. 700 minutes after inoculation the populations in the various tubes were as follows:

	Bacterial population (millions per ml.)
Control	3·467
Succinic acid	10·00
Malic acid	1·413
α-Ketoglutaric acid	4·677
DL-aspartic acid	37·15
DL-glutamic acid	53·70

Determine the effect of the various compounds on the length of the lag phase.

(After DAGLEY, DAWES & MORRISON (1950), *J. gen. Microbiol.*, **4**, 437.)

9.6. An inoculum of 10^6 cells of *Aerobacter aerogenes* in a glucose-ammonium salt medium containing phenol to a concentration of $3{\cdot}4 \times 10^{-3}$ M displayed a lag phase of 100 minutes before logarithmic growth began. After 230 minutes the culture (in the logarithmic phase) had a population of $73{\cdot}55 \times 10^5$ cells. A control tube (no phenol) was given an identical inoculum and grew without lag. At 180 minutes its population was $15{\cdot}85 \times 10^6$ cells and it was still growing exponentially.

From these data, determine whether the concentration of phenol employed exerted any effect on the rate of division of the organism.

9.7. A facultatively anaerobic organism was inoculated into three identical 25 ml. quantities of medium and grown (*a*) with aeration, (*b*) without aeration and (*c*) strictly anaerobically in a Fildes–McIntosh jar. The aerated culture lagged for 55 minutes before active cell division began, but the other two grew immediately and after 250 minutes the populations in the three tubes were (*a*) 446·7, (*b*) 281·8 and (*c*) 63·10 millions of bacteria per ml. If the inoculum per tube was 5×10^7 cells, determine the mean generation times for growth under the three conditions of aerobiosis.

9.8. A medium containing 0·25 g. per litre glucose and an unknown amount of galactose was inoculated with 5×10^5 cells of *Escherichia coli* per ml. medium. No lag was exhibited and the cells grew with a mean generation time of 40 minutes utilizing the glucose. When the glucose was exhausted, the cells adapted themselves to galactose and then grew with a mean generation time

of 45 minutes to attain a stationary population of $3{\cdot}08 \times 10^8$ cells per ml., 6 hours 30 minutes after inoculation.

Determine the length of the phase of adaptation between the two growth cycles and also the amount of galactose present in the medium, given that the relationship between stationary population and sugar concentration is linear up to 0·9 g. per litre of either sugar, at which concentration the stationary population supported is $3{\cdot}7 \times 10^8$ bacteria per ml.

(Glasgow Double Science Course, 1952.)

9.9. What factors influence the size of the bacterial crop that a given medium will support?

After light inoculation with *Escherichia coli* crops of 120 and 510 millions of cells/ml. grew respectively in media containing 240 and 1,020 mg./litre of either glucose or mannose. A medium containing a mixture of the sugars was similarly inoculated with the following results:

Time after inoculation (minutes)	220	260	280	310	340	372	400	440
Cell count (millions/ml.)	69	141	200	224	251	355	490	500

Using a graphical method, find the approximate concentration of each sugar present.

(Leeds Honours Course Finals, 1953.)

9.10. A medium containing $0{\cdot}4 \times 10^{-2}$ M glucose and $7{\cdot}1 \times 10^{-2}$ M citrate was inoculated with cells of *Aerobacter aerogenes*, which had been grown anaerobically on citrate, to give an inoculum population of $6{\cdot}31 \times 10^5$ cells per ml. Determine the length of the lag phase between the phases of growth on glucose and on citrate if, after 400 minutes, the cells were growing logarithmically and utilizing citrate, and the population at this time was 5495×10^5 cells per ml.

The mean generation times of the organism for citrate and glucose under these conditions of unaerated growth are respectively 38 and 80 minutes. The relationship between the concentration of glucose supplied as the sole carbon source and stationary population is linear up to $8{\cdot}6 \times 10^{-3}$ M, at which level the population supported is 518×10^6 cells per ml.

(After Dagley & Dawes (1953), *J. Bact.*, **66**, 259.)

CHAPTER X

OXIDATION-REDUCTION POTENTIALS

OXIDATION may be defined as the loss of electrons and reduction as the gain of electrons. The two processes are complementary, so that whenever one substance is oxidized another must be reduced. A typical oxidation-reduction (O-R) system may be written as

$$\text{Reduced form} \rightleftharpoons \text{Oxidized form} + ne,$$

where n is the number of electrons involved in the reaction.

If an inert metal electrode, such as platinum or silver, is immersed in a solution of an O-R system, a potential difference is set up between the electrons in solution and those in the metal. This gives rise to an *electrode potential* and it can be shown that the value of this potential, designated by E, is given by the equation

$$E = E^\circ + \frac{RT}{nF} \ln \frac{(\text{ox})}{(\text{red})} \quad . \quad . \quad . \quad (10.1)$$

where E° is a special constant for a given system known as the *standard electrode potential* (or standard e.m.f.), R is the gas constant equal to 8·314 absolute joules per degree per mole, T the absolute temperature, n the number of equivalents involved in the reaction, F the faraday (equal to 96,494 coulombs) which is necessary to convert one equivalent of an element to an equivalent of ions, and (ox) and (red) are the activities of the oxidized and reduced forms of the O-R system.

An electrode immersed in a solution of an O-R system constitutes a half-cell, the potential difference of which it is impossible to measure since it is a single electrode; to do so it must be combined with another half-cell, thereby making a complete cell, and the potential difference of the two half-cells measured by connecting them to a potentiometer. Preferably a reference standard half-cell should be used. The hydrogen electrode is such a standard and its potential is arbitrarily taken as zero. The cell reaction is

$$\tfrac{1}{2}H_2 \rightleftharpoons H^+ + e$$

and, by definition, the potential of this system is zero at all temperatures when an inert metal electrode is placed in a solution of unit activity with respect to hydrogen ions, i.e. pH = 0, in equilibrium with gaseous hydrogen at a pressure of 1 atmosphere.

To determine the potential difference of a system it is not necessary to use a hydrogen electrode. Any other half-cell, the potential difference of which has been accurately determined with respect to the hydrogen half-cell, may be used, and in fact the calomel cell is usually more convenient in practice than the hydrogen electrode. The symbol E_h is used for electrode potential to show that the hydrogen electrode is taken as the reference standard. Consequently $E_h = E - E_H$, where E_H is the potential of the hydrogen electrode which, by definition, is zero. Hence the general equation for the potential of an O-R system becomes

$$E_h = E^\circ + \frac{RT}{nF} \ln \frac{(ox)}{(red)} \quad . \quad . \quad (10.1a)$$

At 30°, which is the temperature frequently employed for electrode measurements, the factor $2{\cdot}303RT/nF$ (converting natural logarithms to the base 10) has a value of 0·06 for $n = 1$ and 0·03 for $n = 2$. Thus, for $n = 1$

$$E_h = E^\circ + 0{\cdot}06 \log \frac{(ox)}{(red)} \quad . \quad . \quad (10.2)$$

The general equation reveals the fact that the electrode potential of an O-R system is dependent on a constant, the standard electrode potential, and upon the logarithm of the ratio of the activities of oxidized to reduced forms. With dilute solutions the molar concentrations may be used in place of the activities, i.e. the activity coefficients are assumed to be unity. The potential will be increased, or become more positive, if the proportion of oxidized to reduced form is increased and, conversely, become lower or more negative if the proportion of reduced form becomes greater.

When the system is half-reduced and the ratio of oxidized to reduced activities becomes unity, $E_h = E^\circ$ and hence the standard electrode potential is that of a half-reduced system. E° is of considerable importance because, knowing its value, the electrode potential at any degree of oxidation or reduction of the system can be calculated. Conversely, the degree of oxidation can be obtained

from the measured electrode potential. E° is a measure of the oxidation or reduction intensity of a system and enables a list of O-R systems to be drawn up in order of their standard electrode potentials. Any given system will be capable of being oxidized by a system more positive, i.e. above it on the scale, and in its turn will oxidize any system more negative than itself (below it on the scale). Thus in Fig. 10.1 (*a*) system A will oxidize systems B and C, and B will oxidize C, whereas system C will reduce systems B and A, and B will reduce A. It should be mentioned that a catalyst may be needed in order to bring about the reaction between two such systems. In Fig. 10.1 (*b*) are drawn up some systems of biological interest.

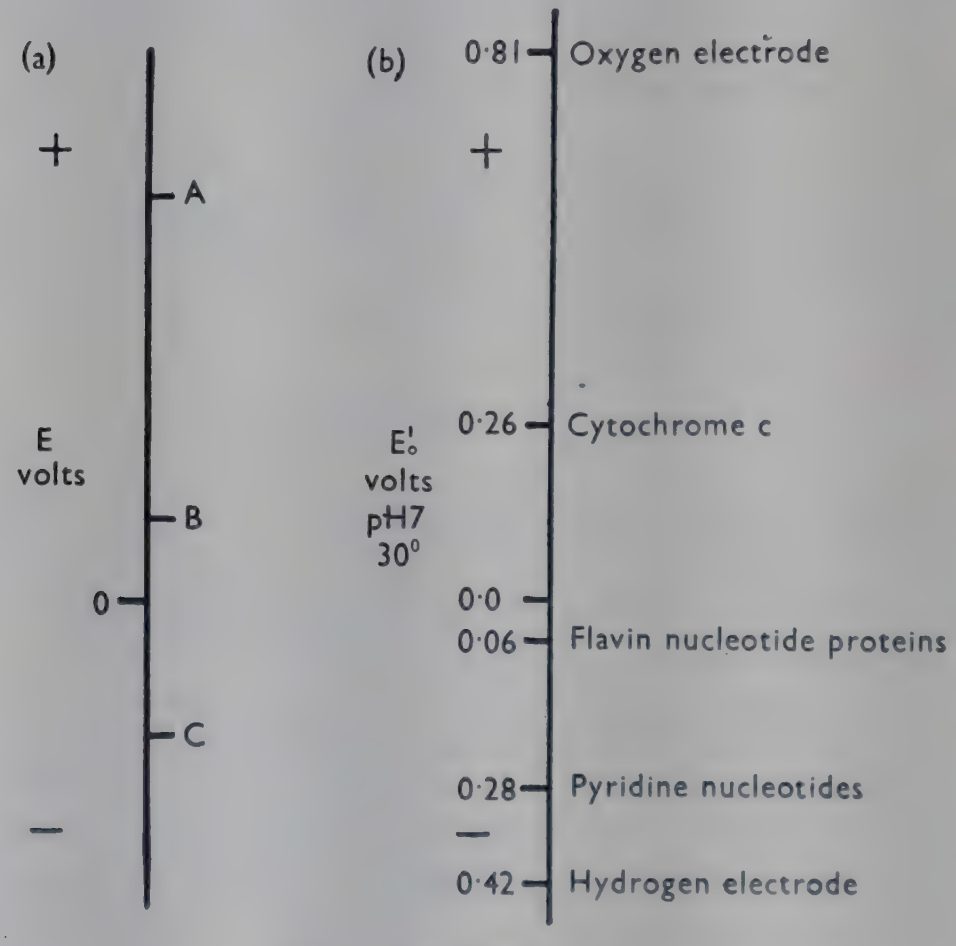


FIG. 10.1

(*a*) Standard electrode potential scale of oxidation-reduction systems.
(*b*) Biological oxidation-reduction systems.

Note that E° refers to measurements at zero pH and, since it is often impracticable to measure certain systems at this pH value,

the term E_0' is introduced to indicate the standard electrode potential at a *stated* pH other than zero.

Example 10.1.—At pH 7·0 E_0' for the methylene blue-leucomethylene blue system is 0·011 volt at 30°. If the measured electrode potential of a solution of this system is 0·065 volt, calculate the percentage of the reduced (leuco) form present.

The reaction for the reduction of methylene blue may be written as

$$MB + H_2 \rightleftharpoons MBH_2.$$

Two hydrogen atoms are involved so that $n = 2$ for this system and

$$E_h = E_0' + \frac{2{\cdot}303RT}{2F} \log \frac{(MB)}{(MBH_2)}$$

$$0{\cdot}065 = 0{\cdot}011 + 0{\cdot}03 \log \frac{(MB)}{(MBH_2)}$$

$$\log \frac{(MB)}{(MBH_2)} = \frac{0{\cdot}054}{0{\cdot}03} = 1{\cdot}8.$$

whence

$$\frac{(MB)}{(MBH_2)} = 63{\cdot}1.$$

Let x be the percentage of MBH_2, then

$$\frac{100 - x}{x} = 63{\cdot}1 \text{ and } x = \underline{1{\cdot}56}.$$

The system, therefore, contains 1·56 per cent. of leucomethylene blue.

Example 10.2.—The riboflavin-leucoriboflavin system has an E_0' value of −0·186 volt at pH 7·0 and 20°. Calculate the oxidation-reduction potential of this system when it contains (*a*) 10 per cent. and (*b*) 80 per cent. of the oxidized form.

The reaction may be written

$$X + H_2 \rightleftharpoons XH_2,$$

where X represents riboflavin and XH_2 its reduced form. Two hydrogen atoms or, in principle, two electrons are involved, hence $n = 2$.

$$E_h = E_0' + \frac{2{\cdot}303RT}{nF} \log \frac{(X)}{(XH_2)}$$

$$= -0{\cdot}186 + \frac{2{\cdot}303 \times 8{\cdot}314 \times 293}{2 \times 96494} \log \frac{(X)}{(XH_2)}$$

$$= -0{\cdot}186 + 0{\cdot}029 \log \frac{(X)}{(XH_2)}.$$

Therefore in (*a*)

$$E_h = -0{\cdot}186 + 0{\cdot}029 \log \frac{10}{90}$$

$$= -0{\cdot}186 + 0{\cdot}029 \log 0{\cdot}1111$$

$$= -0{\cdot}186 + 0{\cdot}029 (\bar{1}{\cdot}0457)$$

$$= -0{\cdot}186 - 0{\cdot}029 \times 0{\cdot}9543$$

$$= -0{\cdot}186 - 0{\cdot}028$$

$$= \underline{-0{\cdot}214 \text{ volt}}.$$

(*b*)

$$E_h = -0{\cdot}186 + 0{\cdot}029 \log \frac{80}{20}$$

$$= -0{\cdot}186 + 0{\cdot}029 \times 0{\cdot}6021$$

$$= -0{\cdot}186 + 0{\cdot}017$$

$$= \underline{-0{\cdot}169 \text{ volt}}.$$

Consider now the reactions between two different O-R systems. If two systems at the same pH but with different potentials are mixed, reaction will occur between them until equilibrium is reached, i.e. until both systems achieve the same potential. To illustrate this, the succinic dehydrogenase and methylene blue systems, as used in the Thunberg tube technique, may be considered. The reactions involved are

$$\text{succinate}'' \rightleftharpoons \text{fumarate}'' + H_2$$

$$MB + H_2 \rightleftharpoons MBH_2$$

$$\text{Net reaction : succinate}'' + MB \rightleftharpoons \text{fumarate}'' + MBH_2$$

The equilibrium constant for this reaction is given by the expression

$$K = \frac{(\text{fum.})(MBH_2)}{(\text{succ.})(MB)} .$$

If 1 ml. of a solution containing succinate and fumarate, both at 0·001 M, together with the enzyme succinic dehydrogenase, are in the evacuated Thunberg tube, and 1 ml. of a methylene blue solution is in the hollow stopper, also 0·001 M with respect to both oxidized and reduced forms, at pH 7·0 and 30°, then for each system $E_h = E_0'$, because both are half-reduced. E_0' for the succinate system is +0·005 volt and for the MB-MBH_2 system +0·011 volt.

When the solutions are mixed by tipping the tube the succinate-fumarate system, having the lower potential, reduces the methylene blue system and is itself oxidized. Consequently the ratio (fum.)/(succ.) increases and the ratio (MB)/(MBH_2) decreases, causing an increase in the potential of the former and a decrease in the potential of the latter until equilibrium is attained, when both are at the same potential, i.e. $E_{h_1} = E_{h_2}$. Therefore

$$E_{0\ \text{fum.-succ.}}' + 0{\cdot}03 \log \frac{(\text{fum.})}{(\text{succ.})} = E_{0\ \text{MB-MBH}_2}' + 0{\cdot}03 \log \frac{(MB)}{(MBH_2)}$$

and $$\Delta E_0' - 0{\cdot}011 - 0{\cdot}005 = 0{\cdot}03 \log \frac{(\text{fum.})(MBH_2)}{(\text{succ.})(MB)} .$$

That is, $$0{\cdot}006 = 0{\cdot}03 \log \frac{(\text{fum.})(MBH_2)}{(\text{succ.})(MB)} .$$

But in the example considered it follows that, since the reaction started with equal concentrations of all four components, (fum.) = (MBH_2) and (succ.) = (MB) at all stages of the reaction. Therefore the above relationship reduces to

$$0{\cdot}006 = 0{\cdot}03 \times 2 \log \frac{(\text{fum.})}{(\text{succ.})},$$

whence $$\log \frac{(\text{fum.})}{(\text{succ.})} = 0{\cdot}10$$

and $$\frac{(\text{fum.})}{(\text{succ.})} = \frac{(MBH_2)}{(MB)} = 1{\cdot}26.$$

Let x be the percentage of MBH_2, then

$$\frac{x}{100 - x} = 1{\cdot}26 \text{ and } x = 55{\cdot}8.$$

Thus, at equilibrium, the dye would be 55·8 per cent. reduced, showing that under these conditions the amount of reduction occurring is quite small, namely from 50 to 55·8 per cent.

Free Energy of Oxidation-Reduction Reactions.

In the case just discussed, it was seen that

$$\Delta E_0' = 2{\cdot}303 \frac{RT}{nF} \log \frac{(\text{fum.})(MBH_2)}{(\text{succ.})(MB)}.$$

But the equilibrium constant, K, of this reaction is equal to (fum.)(MBH_2)/(succ.)(MB) and this enables the following general expression to be derived :

$$\Delta E_0' = \frac{RT}{nF} \ln K$$

or $$nF\Delta E_0' = RT \ln K.$$

But from thermodynamics we have the relationship between the standard free energy change of a reaction, ΔG°, and the equilibrium constant (equation 3.9) :

$$\Delta G^\circ = -RT \ln K,$$

from whence it follows that

$$\Delta G^\circ = -nF\Delta E_0'. \quad . \quad . \quad . \quad (10.3)$$

This means that the standard free energy change of the reaction between two O-R systems may be calculated from the difference in E_0' values. In similar manner it is possible to calculate the free energy change for the two individual systems :

succ.-fum. $\quad \Delta G_1^\circ = -nF\Delta E'_{0(\text{succ.-fum.})}$

$\text{MB-MBH}_2 \quad \Delta G_2^\circ = -nF\Delta E'_{0(\text{MB-MBH}_2)}$

and the free energy change for the combined systems is then

$$\Delta G^\circ = \Delta G_2^\circ - \Delta G_1^\circ.$$

The value of ΔG° in this calculation will be in joules, therefore to express it in calories it is necessary to divide by 4·184. Hence the free energy change of a reaction is given by the general equation

$$\Delta G = -nF\Delta E \quad . \quad . \quad . \quad (10.3a)$$

where ΔE is the potential difference between the participating systems, provided n is the same for these two systems.

Example 10.3.—Calculate the free energy change of the reaction

lactate + acetaldehyde ⇌ pyruvate + ethanol.

At pH 7·0, E_0' for the pyruvate-lactate system is −0·18 volt and for the acetaldehyde-ethanol system is −0·16 volt. DPN is the coenzyme for both systems.

$$\Delta G = -nF\Delta E_0'.$$

For this reaction $n = 2$ and $\Delta E_0' = -0{\cdot}16 - (-0{\cdot}18) = +0{\cdot}02$ volt.

Therefore $\quad \Delta G = \dfrac{-2 \times 96494 \times 0{\cdot}02}{4{\cdot}184}$ calories

$$= \underline{-923 \text{ calories.}}$$

Potentiometric Titration.

If the course of a reaction between two O-R systems is followed potentiometrically a curve relating the potential difference to the percentage oxidation or reduction can be constructed (Fig. 10.2 (*a*) and (*b*)).

Curve A shows the effect of adding a strong oxidizing agent to the reduced form of an O-R system, and curve B the reverse process, reduction of the oxidized form of the O-R system. At 50 per cent. oxidation $E_h = E^\circ$ and there is a point of inflexion in the curve. The slope of the curve at the inflexion point is directly related to

the number of electrons involved in the process and is therefore useful in determining the type of reaction under investigation. Fig. 10.2 (*a*) shows the titration curve for a system where $n = 1$ and it will be noticed that the curve is steeper than in Fig. 10.2 (*b*),

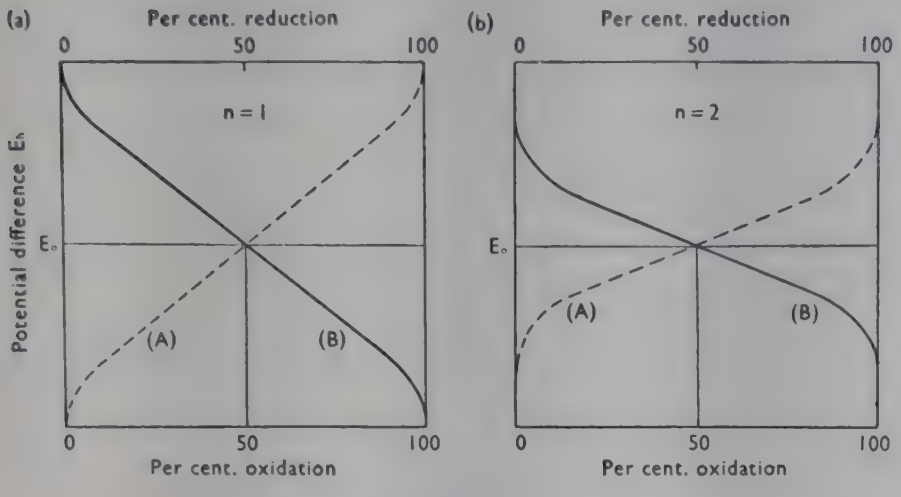
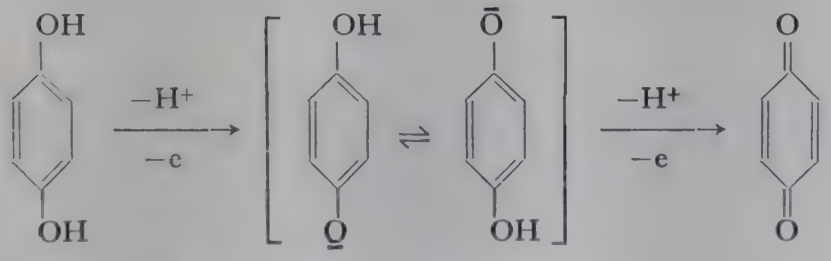


FIG. 10.2

Potentiometric titration curves for oxidation-reduction systems (*a*) where $n = 1$ and (*b*) where $n = 2$.

where $n = 2$. Some biological O-R systems which involve an overall transfer of two electrons may actually occur in two distinct one-electron steps, e.g. the reduction of flavins via semi-quinone formation. Semi-quinone formation has been investigated by Michaelis. Hydroquinone is oxidized to quinone via the intermediate resonance-stabilized semi-quinone structure



Semi-quinone resonance hybrid

This behaviour is revealed in the potentiometric titration curve, as in Fig. 10.3 for the titration of reduced pyocyanine with ferricyanide where two distinct curves are evident, each corresponding to a one-electron transfer. This is to be compared with the smooth curve for two-electron transfer where no semi-quinone formation occurs (Fig. 10.2 (*b*)). The whole question of one-electron and

two-electron transfer reactions has been reviewed by Westheimer (1954).

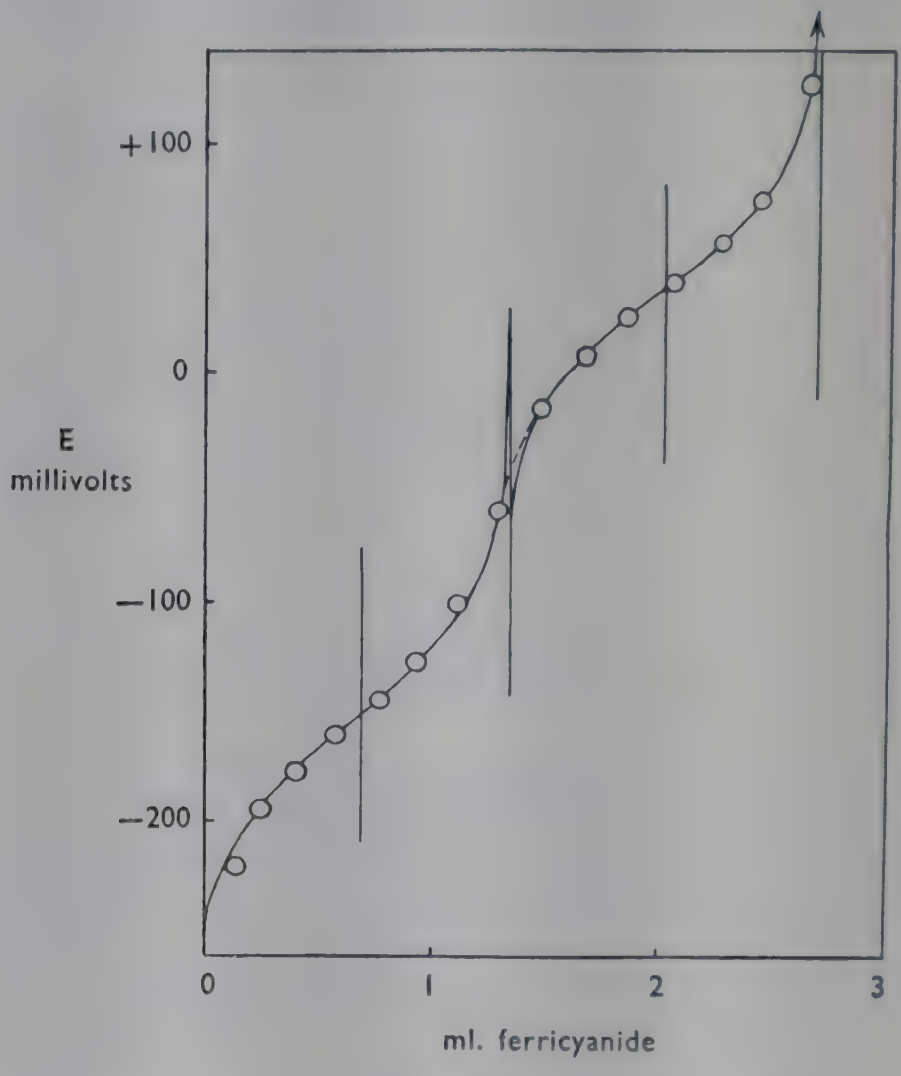


FIG. 10.3

Potentiometric titration of reduced pyocyanine with ferricyanide ion at pH 1·82. Note the two oxidation steps corresponding to one-electron transfers. At 50 per cent. oxidation the semi-quinone form of pyocyanine is present. (FRIEDHEIM & MICHAELIS, 1931.)

Effect of pH on Electrode Potentials.

In deriving the general equation for electrode potential, a constant pH was assumed. Should the pH change, however, complications may arise due to changes in the ionization of components of the system; for example, either or both of oxidized and reduced forms may be ionized. This is frequently the case with biological systems.

Consider the general case of the reduced form being ionized and the oxidized form unionized, namely:

$$\text{red}' \rightleftharpoons \text{ox} + \text{e}.$$

The E_h of this system is given by the equation

$$E_h = E^\circ + \frac{RT}{nF} \ln \frac{(\text{ox})}{(\text{red}')} \quad . \quad . \quad (10.1a)$$

where $n = 1$. Furthermore, since

$$\text{Hred} \rightleftharpoons \text{H}^+ + \text{red}'$$

the dissociation constant of this acid, K_a, is

$$K_a = \frac{(\text{H}^+)(\text{red}')}{(\text{Hred})} \quad . \quad . \quad . \quad (10.4)$$

The total concentration of the reduced form (red) will be the sum of the ionized and non-ionized fractions, i.e.

$$(\text{red}) = (\text{red}') + (\text{Hred}) \quad . \quad . \quad (10.5)$$

By combining equations 10.4 and 10.5 we obtain

$$(\text{red}') = (\text{red}) \frac{K_a}{K_a + (\text{H}^+)} \quad . \quad . \quad (10.6)$$

and substituting this in the general equation 10.1a we obtain

$$E_h = E^\circ + \frac{RT}{F} \ln \frac{(\text{ox})}{(\text{red})} - \frac{RT}{F} \ln \frac{K_a}{K_a + (\text{H}^+)} . \quad (10.7)$$

This is the general electrode equation which holds for all values of pH in the case of the monobasic acid considered. If the pH is constant, then the last term in equation 10.7 becomes a constant and the equation can be contracted to

$$E_h = E_0' + \frac{RT}{F} \ln \frac{(\text{ox})}{(\text{red})} \quad . \quad . \quad . \quad (10.1b)$$

where E_0' is the standard electrode potential at the given constant pH value.

If the reduced form should be a dibasic acid having primary

and secondary dissociation constants K_{a_1} and K_{a_2} respectively, the expression becomes:

$$E_h = E^\circ + \frac{RT}{2F} \ln \frac{(ox)}{(red)} + \frac{RT}{2F} \ln [(H^+)^2 + K_{a_1}(H^+) + K_{a_1}K_{a_2}] \quad . \quad . \quad . \quad . \quad (10.8)$$

At low pH values $(H^+)^2$ is great compared with $K_{a_1}(H^+)$ and $K_{a_1}K_{a_2}$ so that the latter two terms may be neglected and

$$E_h = E^\circ + \frac{RT}{2F} \ln \frac{(ox)}{(red)} + \frac{RT}{2F} \ln (H^+)^2 \quad . \quad (10.9)$$

and at 30°

$$E_h = E^\circ + \frac{RT}{2F} \ln \frac{(ox)}{(red)} - 0{\cdot}06 \text{ pH} \quad . \quad . \quad (10.10)$$

When (ox) = (red), E_h varies with pH in a linear manner and the slope of the E_h (or, in effect, E_0') versus pH curve will be $-0{\cdot}06$. At higher pH values, where $K_{a_1}(H^+)$ is much greater than $(H^+)^2$ or $K_{a_1}K_{a_2}$ and the latter two terms may be neglected, the slope of the line will be $-0{\cdot}03$. Extrapolation of the lines of slope $-0{\cdot}06$ and $-0{\cdot}03$ to their point of intersection gives a value for pK_{a_1} of the dibasic acid. At high pH values $K_{a_1}K_{a_2}$ is the only significant term in the last term of equation 10.8 and the pH curve then has zero slope. This enables the value of pK_{a_2} to be obtained by extrapolation of the lines of slope $-0{\cdot}03$ and 0 to their point of intersection. Fig. 10.4 illustrates the pH dependence curve for a dibasic ionizing system. It will be obvious that this is not a very exact method for obtaining pK_a values.

The variation of E_h with pH in the linear portions of the pH dependence curve may be expressed, at 30°, by the equation

$$E_h = E^\circ + \frac{0{\cdot}06}{n} \log \frac{(ox)}{(red)} - \frac{0{\cdot}06a}{n} \text{ pH} \quad . \quad (10.11)$$

where n is the number of electrons involved and a the number of hydrogen ions released in the oxidation process. The symbol $-\Delta E_h/\Delta \text{pH}$ is used to denote the pH dependence of E_h and is often encountered in tables of oxidation-reduction potentials.

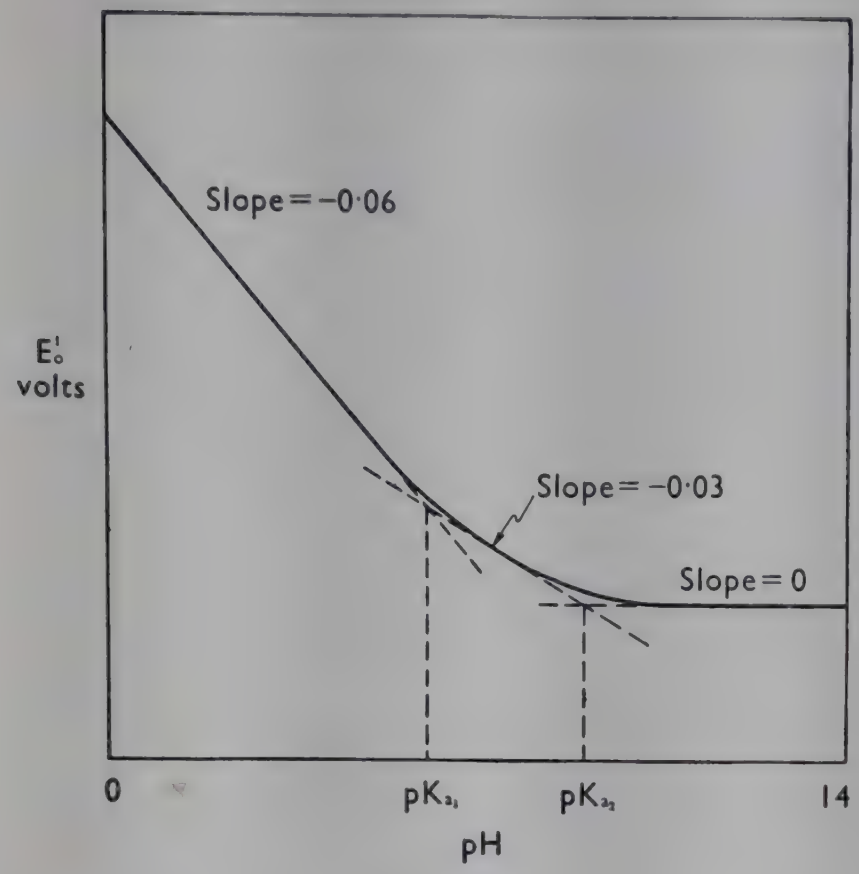


FIG. 10.4

Graph of E_0' values for a dibasic ionizing oxidation-reduction system plotted against pH and showing method of obtaining values for pK_{a_1} and pK_{a_2} by extrapolation of lines of different slope to their points of intersection.

The rH Scale.

An alternative method of expressing an oxidation-reduction reaction, and one which has obvious advantages when considering biochemical O-R systems, is

$$AH_2 \rightleftharpoons A + H_2.$$

This enables a hydrogen scale of oxidation-reductions to be used since the oxidized and reduced forms are related to hydrogen by the above equation. rH is defined as

$$rH = -\log (H_2) \quad . \quad . \quad . \quad (10.12)$$

where (H_2) is the hydrogen pressure in atmospheres (compare with $pH = -\log (H^+)$). The value of rH is obtained from the general electrode equation (10.1a) and the electrode reaction of the hydrogen half-cell :

$$\tfrac{1}{2}H_2 \rightleftharpoons H^+ + e.$$

Thus $$E_h = E^\circ + \frac{RT}{F} \ln \frac{(H^+)}{\sqrt{(H_2)}} \quad . \quad . \quad . \quad (10.13)$$

But for the hydrogen electrode $E_h = 0$ when (H^+) and (H_2) are both unity, hence E° must also be zero. Therefore

$$E_h = 2{\cdot}303 \frac{RT}{F} \log \frac{(H^+)}{\sqrt{(H_2)}} \quad . \quad .(10.13a)$$

and since $pH = -\log (H^+)$ and $rH = -\log (H_2)$

$$E_h = 2{\cdot}303 \frac{RT}{F} \left(\frac{rH}{2} - pH\right)$$

or $$E_h = 2{\cdot}303 \frac{RT}{2F} (rH - 2pH). \quad . \quad . \quad (10.14)$$

At 30° this becomes

$$E_h = 0{\cdot}03(rH - 2pH) \quad . \quad . \quad .(10.14a)$$

When the pressure of hydrogen gas is 1 atmosphere, $rH = 0$ and

$$E_h = -0{\cdot}06pH \quad . \quad . \quad . \quad . \quad (10.15)$$

that is the potential varies only when there is an alteration in pH, and therefore at pH 7 $E_h = -0{\cdot}42$ volt. This is the basis for the use of the hydrogen electrode in the determination of pH values.

Dixon (1949) has advocated the use of the rH scale rather than the scale of electrode potentials since it confers certain advantages. He has developed a series of useful rules which has no counterpart on any other scale. The rH of hydrogen gas at 1 atmosphere pressure is zero and this is taken as the datum line for the rH scale. The free energy of hydrogen at this pressure is also zero; this follows from the relationship $\Delta G = -nFE_h$, since E_h is zero for the standard hydrogen electrode. The scale extends from the hydrogen datum line to oxygen at 1 atmosphere pressure, which is at $rH = 41$. All other O-R systems are placed at such positions on the scale that their distance is proportional to the free energy change of the O-R reaction. Most biological systems lie within the range rH 0-25. For convenience, a calorie scale is used in conjunction with the rH one; rH is related to the free energy by the equation

$$\Delta G = -2{\cdot}303RT \times rH \text{ calories} \quad . \quad . \quad (10.16)$$

At 30° this becomes

$$\Delta G = -1380 \text{ rH calories} \quad . \quad . \quad .(10.16a)$$

Here the gas constant R has the value of 1·987 calories per degree per mole. Note particularly that equation 10.16 applies to systems involving two electrons, since they are being balanced against $H_2 \rightleftharpoons 2H^+ + 2e$ and $rH = -\log(H_2)$. Where $n = 1$, however, the system is being balanced against $\frac{1}{2}H_2 \rightleftharpoons H^+ + e$ and the free energy is related to the work done in expanding a *half* mole of hydrogen from 1 atmosphere to the required pressure. Hence for cases where $n = 1$

$$\Delta G = -2{\cdot}303RT \times \frac{rH}{2} \quad . \quad .(10.16b)$$

The free energy change for the reaction between any two O-R systems is given by

$$\Delta G = -2{\cdot}303RT\Delta rH \quad . \quad . \quad .(10.16c)$$

where ΔrH is the difference between the rH values of the two systems. This applies whatever the percentage reduction of the systems. At equilibrium ΔG is, of course, zero. The standard free energy change of the reaction, $\Delta G°$, is a property of the reaction itself and, as seen in Chapter III, is defined as the free energy change when all the reactants are at unit activity. If we assume the activity coefficients of all reactants to be unity (an assumption often made with biological systems but by no means always justified), then $\Delta G° = \Delta G$ when the concentrations are 1 M. Under these conditions (ox) = (red) for each system and this corresponds to the midpoint of the rH versus percentage reduction curves, designated by $rH°$ (compare with $E°$). Hence:

$$\Delta G° = -2{\cdot}303\ RT\Delta rH° \quad . \quad .(10.16d)$$

and comparing with equation 3.9 it will be seen that

$$\log K = \Delta rH° \quad . \quad . \quad . \quad (10.17)$$

where K is the equilibrium constant of the reversible O-R reaction. This affords an extremely simple method of obtaining the equilibrium constant of the reaction between two O-R systems and is very useful in the study of linked dehydrogenase systems.

Applying the van't Hoff Isochore (equation 4.8), to rH° values, we have

$$\Delta H = 2{\cdot}303\ RT^2 \frac{d(\mathrm{rH}^\circ)}{dT} \quad . \quad . \quad (10.18)$$

$$= 418100 \frac{d(\mathrm{rH}^\circ)}{dT} \text{ calories at } 30^\circ,$$

so that the heat of reaction, ΔH, can be determined provided the rate of change of rH° with temperature is known.

As previously discussed in connexion with electrode potentials, the rH expressions become more complex if ionization and pH changes occur. The reader is referred to Dixon's book for full treatment of these cases. Dixon denotes the half-reduced ionizing system by the symbol r'H. r'H is related to E_0' by the equation

$$\mathrm{r'H} = \frac{2E_0'F}{2{\cdot}303RT} + 2\mathrm{pH} \quad . \quad . \quad (10.19)$$

and rH° is related to E° by

$$\mathrm{rH}^\circ = \frac{2E^\circ F}{2{\cdot}303RT} \quad . \quad . \quad . \quad . \quad (10.20)$$

which are derived from equation 10.14 and the fact that E° refers to pH = 0.

Example 10.4.—Determine the electrode potential of the hydrogen electrode at 30° in equilibrium with a partial pressure of 10^{-5} atmosphere of hydrogen at (*a*) pH 6·0 and (*b*) pH 7·5.

Using equation 10.14, we have:

$$E_h = \frac{RT}{2F} \times 2{\cdot}303(\mathrm{rH} - 2\mathrm{pH})$$

$$\mathrm{rH} = -\log (H_2) = -\log 10^{-5} = 5.$$

Therefore at pH 6·0 and 30°

$$E_h = \frac{8{\cdot}314 \times 303 \times 2{\cdot}303}{2 \times 96494}(5 - 2 \times 6)$$

$$= -0{\cdot}03 \times 7$$

$$= -0{\cdot}21 \text{ volt.}$$

At pH 7·5, $E_h = 0{\cdot}03(5 - 2 \times 7{\cdot}5)$

$$= -0{\cdot}30 \text{ volt.}$$

Example 10.5.—The lactic dehydrogenase system of muscle requires DPN as coenzyme and has an r'H value of 8 at pH 7·5. The reduced coenzyme is oxidized by diaphorase and the DPNH-DPN system has r'H = 4 at the same pH value. All measurements are at 30°. Determine the free energy change

on interaction of the two systems if all reactants are at the same initial concentration.

$$\triangle r'H = 8 - 4 = 4$$

and

$$\triangle G = -2{\cdot}303\ RT\triangle r'H$$
$$= -1380 \times 4$$
$$= \underline{-5{,}520 \text{ calories.}}$$

Example 10.6.—The E_o' values for the malic-oxaloacetic acid and methylene blue-leucomethylene blue systems are $-0{\cdot}102$ and $+0{\cdot}011$ volt respectively at pH 7 and 30°. Determine the r'H values for these systems and the free energy change and equilibrium constant of the reaction between them. Assume that all reactants are present at the same initial concentration.

$$r'H = \frac{2E_o'F}{2{\cdot}303RT} + 2pH.$$

For the malic-oxaloacetic system

$$r'H = -\frac{2 \times 0{\cdot}102 \times 96494}{2{\cdot}303 \times 8{\cdot}314 \times 303} + 14$$
$$= \frac{-0{\cdot}102}{0{\cdot}03} + 14 = -3{\cdot}4 + 14$$
$$r'H = \underline{+10{\cdot}6.}$$

For the MB-MBH_2 system

$$r'H = \frac{0{\cdot}011}{0{\cdot}03} + 14 = 0{\cdot}36 + 14$$
$$r'H = \underline{+14{\cdot}36.}$$

The free energy change is given by equation 10.16c.

$$\triangle G = -2{\cdot}303RT\triangle r'H$$
$$= -2{\cdot}303 \times 1{\cdot}987 \times 303 \times (14{\cdot}36 - 10{\cdot}6)$$
$$= -1380 \times 3{\cdot}76$$
$$\triangle G = \underline{-5189 \text{ calories.}}$$

The equilibrium constant of the reaction is obtained from equation 10.17 when, as already seen,

$$\log K = \triangle r'H = 3{\cdot}76$$

and

$$K = \frac{(\text{oxaloacetate})(MBH_2)}{(\text{malate})(MB)} = \underline{5{,}754.}$$

This question specifically asked for the calculation of r'H values, but if these had not been required, $\triangle G$ for the reaction could have been obtained in an alternative manner by use of equation 10.3a.

$$\triangle G = -nF\triangle E.$$

In this case $n = 2$ and $\triangle E = 0{\cdot}011 - (-0{\cdot}102) = +0{\cdot}113$ volt.

Therefore $\triangle G = -2 \times 96494 \times 0{\cdot}113$ joules

$$= \frac{-2 \times 96494 \times 0{\cdot}113}{4{\cdot}184} \text{ calories}$$
$$= \underline{-5{,}212 \text{ calories.}}$$

The agreement between this and the previously derived value is fairly reasonable.

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PROBLEMS

10.1. Determine the percentage of the reduced form of cresyl violet at pH 7·0 when the oxidation-reduction potential is (*a*) −0·128 volt and (*b*) −0·179 volt. E_0' for cresyl violet at pH 7·0 is −0·166 volt and n is 2. Assume a temperature of 30° throughout.

10.2. What is the ratio of reduced to oxidized forms of the pigment pyocyanine if the measured oxidation-reduction potential of the system is −0·025 volt? E_0' for the reaction is −0·034 volt at the same temperature (25°) and pH. ($n = 2$ for pyocyanine.)

10.3. The lactic dehydrogenase system, catalysing the oxidation of lactic to pyruvic acid, has an E_0' value of −0·180 volt at 35° and pH 7·01. What will be the potential of this system when the oxidation has gone to 95 per cent. completion?

10.4. The dye 2:6 dichlorophenolindophenol is used for the quantitative estimation of ascorbic acid. The respective E_0' values at pH 7·0 and 30° are 0·217 and 0·060 volt. What is the potential of the dye when it has been 99·8 per cent. reduced by ascorbic acid?

If equal concentrations of ascorbic and dehydroascorbic acids are reacted with the same concentration of oxidized and reduced forms of the dye, what will be the final oxidation-reduction potential of the system?

10.5. The formic hydrogenlyase system of *Escherichia coli* catalyses the reaction

$$HCOOH \rightleftharpoons H_2 + CO_2.$$

At 30° and pH 7 the E_0' value is −0·420 volt. What is the oxidation-reduction potential of this system when the equilibrium constant of the reaction is 311·6?

10.6. A Thunberg experiment is carried out to follow the activity of a lactic dehydrogenase preparation. In the tube is placed 3 ml. of a solution containing lactate and pyruvate (both at 0·002 M concentration) and the enzyme, while the side arm contains 1 ml. of a mixture of methylene blue and leucomethylene blue in equal concentration at 0·006 M. Determine the percentage oxidation of lactate after mixing the solutions and allowing to attain equilibrium. The

experiment is conducted at 35° and pH 7·0, under which conditions E_0' for the lactate-pyruvate system is −0·180 volt and E_0' for the methylene blue-leuco-methylene blue system is 0·011 volt.

10.7. The following E_0' values were obtained for ascorbic acid at different pH values and at 30°:

pH	1·05	2·16	3·04	4·00	5·19	6·32	7·24	8·57
E_0' (volt)	+0·326	+0·260	+0·209	+0·154	+0·115	+0·078	+0·051	−0·012

From these figures deduce graphically the effect of pH on the course of the oxidation of ascorbic acid. Obtain a value for the standard electrode potential, $E°$, of ascorbic acid.

(After BALL (1937), *J. biol. Chem.*, **118**, 219.)

10.8. The following standard electrode potentials have been determined at 25°.

System:	$Fe-Fe^{2+}$	$Fe^{2+}-Fe^{3+}$	$Fe(CN)_6^{4'}-Fe(CN)_6^{3'}$
$E°$ (volt):	−0·44	+0·77	+0·36

Determine the rH values and the standard free energy changes of these systems.

10.9. Determine the percentage oxidation of thionine if the electrode potential measured at 30° and pH 7·0 is 0·10 volt. E_0' for thionine is 0·063 volt under the same conditions. What is the r′H value for thionine at pH 7·0 and 30°?

10.10. The enzymes lactic dehydrogenase and alcohol dehydrogenase are coenzyme-linked by diphosphopyridine nucleotide (DPN). At pH 7·5 and 35°

$$\text{lactate}' \rightleftharpoons \text{pyruvate}' \quad r'H = 8{\cdot}0$$
$$\text{ethanol} \rightleftharpoons \text{acetaldehyde} \quad r'H = 6{\cdot}8$$

If equal concentrations of all reactants are mixed in the presence of DPN and the two enzymes, determine the free energy change of the reaction.

What percentage of lactate will remain at equilibrium?

10.11. β-Hydroxybutyric acid, which is formed during ketogenesis, is oxidized by the kidneys and muscles. The first step in its oxidation is acetoacetic acid. The hydrogen is transferred by means of DPN and flavoprotein to cytochrome *a*, which may be assumed to be maintained in the half-reduced state by the cell. If equal concentrations of β-hydroxybutyrate and acetoacetate are present initially, determine the free energy liberated by the oxidation of β-hydroxybutyrate and reduction of cytochrome *a*. E_0' for the β-hydroxybutyrate-acetoacetate system is −0·293 volt and for cytochrome *a* is 0·290 volt at pH 7·5 and 37°. Reduced cytochrome *a* reacts with atmospheric oxygen under the influence of the enzyme cytochrome oxidase. Assuming the potential E_0' of atmospheric oxygen to be 0·80 volt, determine the energy liberated by the final reaction with oxygen.

10.12. The following data were obtained in an experiment in which 0·002 M ascorbic acid was titrated with potassium ferricyanide (0·04 M) at 30°. The acid was in 0·1 M acetate buffer of pH 4·581 and a small quantity of thionine

was added to act as a mediator for the reaction of ascorbic acid with the electrode. (This is necessary because otherwise ascorbic acid reacts very sluggishly with the electrode.)

Per cent. oxidation	*Observed* E_h volts
25·37	+0·1224
35·43	+0·1284
45·50	+0·1338
50·55	+0·1364
60·46	+0·1417
70·79	+0·1478
80·75	+0·1552
90·79	+0·1670
95·80	+0·1776

Determine a value for E_0' at pH 4·581 and also the number of electrons involved in the oxidation process at this pH value.

(After BALL (1937), *J. biol. Chem.*, **118**, 219.)

10.13. The overall reaction of biological oxidations may be represented as

$$H_2 + \tfrac{1}{2}O_2 \rightleftharpoons H_2O$$

and the standard free energy of this reaction is −56,560 calories. Use this information to deduce the oxidation-reduction potential of the oxygen-activating system catalysing this final reaction at pH 7·0 and 30°. Assume the pressure of atmospheric oxygen to be 0·2 atmosphere.

Note.—in aqueous solutions the concentration of H_2O may be taken as unity.

10.14. The oxidation-reduction potentials of the haemoglobin-methaemoglobin system have been investigated at various pH values at 30°. The following E_0' values were obtained in such a study:

pH	5·08	5·46	5·85	6·04	6·06	6·12
E_0' volt	0·1673	0·1657	0·1679	0·1681	0·1671	0·1661
pH	6·22	6·36	6·76	7·48	7·56	7·63
E_0' volt	0·1654	0·1615	0·1507	0·1150	0·1126	0·1009
pH	8·50	8·64	8·72	8·75	9·18	
E_0' volt	0·0554	0·0517	0·0444	0·0417	0·0202	

From these data determine the pK value of the system and hence the apparent acidic dissociation constant. What can you deduce about the oxidation of haemoglobin from the E_0'-pH dependence curve?

(After TAYLOR & HASTINGS (1939), *J. biol. Chem.*, **131**, 649.)

10.15. Cytochrome *a* provides a one electron oxidation-reduction system with a standard potential, E_0', at pH 7 of about +0·29 volt.

$E°$ for the oxygen reaction:

$$O_2(g) + 4H^+ + 4e \rightarrow 2H_2O$$

is +1·23 volts at 25° C.

Compute the electrode potential for this reaction at pH 7. Compute the theoretical pressure of oxygen required to maintain the cytochrome *a* system 99·9 per cent. in the oxidized form at 25° C.

(After BALL (1939), *Symposium Quant. Biol.*, **7**, 100. Harvard Medical Sciences 201 ab.)

CHAPTER XI

ISOTOPES IN BIOCHEMISTRY

Some of the most spectacular advances in biochemistry during the last twenty years have stemmed from the application of isotopes to the study of metabolic processes. Compounds have been 'labelled' or 'tagged' by the incorporation of either stable or radioactive isotopes into their molecules and then the subsequent fate of the molecules studied by tracing the movements of the labelled atoms.

The major use of labelled compounds has been to follow metabolic pathways. The appearance of the labelling in some isolated product of a metabolic sequence and the determination of its position in the molecule by suitable degradative procedures has afforded evidence for the existence of certain pathways. The feeding of labelled compounds and their incorporation in the animal body has demonstrated that bodily components are not necessarily inert simply because their concentration remains constant. The classical researches of Schoenheimer and Rittenberg paved the way to the concept of dynamic equilibria in the body tissues. Isotopes have also been used to determine the rates of reactions in living organisms where, despite the constancy of concentration of the bodily components, continual metabolic activity, termed 'turnover', occurs and can be measured by certain isotopic techniques.

Selected aspects of the theoretical background to isotopic tracer work will now be considered.

Stable Isotopes.

The atomic number of an atom, i.e. the number of protons in the nucleus, determines its chemical properties. Almost all known elements exist as mixtures of atoms having two or more different atomic weights. Such atoms, having identical atomic number but different atomic mass, are termed isotopes of the element. Any sample of the element consists of a mixture of isotopes and the

observed chemical atomic weight is therefore the average weight of the mixture of different atoms and depends upon the abundance of each isotope in nature. Thus chlorine has isotopes of mass 35 and 37 in such proportion that the determined atomic weight is almost 35·5, and carbon consists of isotopes of mass 12 and 13 in the proportion of 99·3 to 0·7.

Isotopes have almost identical chemical properties yet may often be separated by difference in physical properties such as rates of diffusion and evaporation. In the case of deuterium, the isotope of hydrogen with mass 2, the situation is very favourable because it has a mass 100 per cent. greater than hydrogen, and heavy water, D_2O, may actually be estimated by the difference in its density compared with ordinary water. If by a suitable enrichment process the proportion of one of the less abundant isotopes of an element can be increased, then a 'labelled' element is produced and compounds containing the element will similarly be labelled. Alteration of the natural abundance ratio is achieved by the same physical techniques that are used for the separation of isotopes, and of these fractional distillation and electrolysis are the main methods used.

The determination of the amounts of stable isotopes present in an element necessitates use of the mass spectrometer for all cases excepting deuterium. The mass spectrometer produces a beam of rays of positively charged gaseous ions of homogeneous energy. By the application of electrostatic and magnetic fields these ions are separated according to their mass, which determines the trajectory. An electrometer guarded by a slit constitutes the measuring device. Application of a potential causes the mass spectra produced to traverse the slit and each ion beam in turn enters the electrometer, where the ionization current is measured. Information is thereby obtained as to the amount of the element of a particular mass present. It is on this type of determination that the use of stable isotopes in biochemistry depends. The abundance of the labelling isotope as compared with the normal abundance, in the labelled starting material and in the product of the reaction, is measured.

The degree of labelling of a compound is always indicated by the *atom per cent. excess*. If an element normally contains y atoms of a particular, say heavy, isotope per 100 atoms of the element, then there will be $100 - y$ atoms of the other isotope or isotopes

and the normal abundance is y atom per cent. Suppose that this abundance is increased to z atom per cent. in the process of labelling. The difference between the enriched and normal abundance, $z - y$, is termed the atom per cent. excess of the heavy isotope. Suppose that during the course of an experiment the labelled material is diluted n times with the element possessing the normal abundance ratio. For every 100 atoms of labelled element there will be a total of $100n$ atoms of the element. Of these, z atoms of the heavy isotope are derived from the enriched material and $y(n - 1)$ atoms of heavy isotope are derived from the diluting material. The resultant element therefore contains $z + y(n - 1)$ atoms of heavy isotope in a total of $100n$ atoms, i.e. $(z + y(n - 1))/n$ atom per cent., and the atom per cent. excess of the isotope is

$$\frac{z + y(n-1)}{n} - y = \frac{z - y}{n} .$$

Since $z - y$ was the atom per cent. excess of the starting material, comparison of the atom per cent. excess of starting and final material gives a measure of the dilution factor n.

The use of stable isotopes has largely been superseded by radioactive ones, wherever there is a choice between the two, on account of the easier methods of measurement and their greater sensitivity. In the case of nitrogen and oxygen, no suitable radioactive isotopes exist and the stable isotopes ^{15}N and ^{18}O are used. Stable isotopes possess the advantage that they are permanent and do not disintegrate with time and furthermore produce no radiation effects on the tissues. Combination of both stable and radioactive isotopes permits the double labelling of a molecule, so that, for example, two different carbon atoms of a molecule may be labelled with stable ^{13}C and radioactive ^{14}C respectively.

Radioactive Isotopes.

Radioactive isotopes for tracer studies may be prepared artificially from non-radioactive elements by means of either the cyclotron or the atomic pile. In this manner considerable quantities of radioactive isotopes have been made available for

biochemical and medical research. Radioactive isotopes disintegrate spontaneously, giving rise to new elements and emitting radiation or particles or both. The emissions may be α, β or γ rays; α rays are helium nuclei (He^{++}) with mass 4, β rays are either positive or negative electrons and γ rays are electromagnetic radiations similar in character to X-rays. From the isotopes used in tracer work, β and γ radiation only are produced, whereas α particles are characteristic of naturally occurring radioactive elements such as radium.

Artificially produced isotopes which have been widely used as tracers include ^{14}C, ^{24}Na, ^{32}P, ^{35}S, ^{40}K, ^{59}Fe and ^{131}I. These all emit negatively charged β particles, and ^{24}Na, ^{59}Fe and ^{131}I additionally emit γ radiation. In the decay process the elements undergo transmutation and the new element product of the process is non-radioactive. For example, ^{14}C is converted to ^{14}N, ^{32}P to ^{32}S and ^{40}K to a mixture of ^{40}Ca and ^{40}A. The conversion has a negligible effect on reactions being followed by the tracers, because the radioactive isotopes form but a minute proportion of the number of non-radioactive atoms of the same element present in the labelled compound.

A radioactive isotope disintegrates at a rate which is a function only of the constitution of its nucleus and which cannot be altered in any way by chemical or physical means. As already seen in Chapter V, the radioactive disintegration process is a first order reaction and the decay constant (rate constant) k is given by the expression

$$k = \frac{2{\cdot}303}{t} \log \frac{n_0}{n} \quad . \quad . \quad . \quad (5.5)$$

where n_0 is the number of atoms of an element at time $t = 0$ and n the number after time t. The half-life period, $t_{\frac{1}{2}}$, or time required for the concentration of the decomposing element to reach half its original concentration, is expressed by equation 5.6

$$t_{\frac{1}{2}} = \frac{0{\cdot}693}{k} \quad . \quad . \quad . \quad . \quad (5.6)$$

Decay constants can be conveniently obtained by the use of semi-logarithmic graph paper, i.e. paper with one axis marked out proportionally to the logarithms of numbers and the other marked

out linearly. A straight line through points $2n,t_1$ and n,t_2, where $2n$ and n are the number of atoms (log axis) at times t_1 and t_2, gives the half-life period $(t_2 - t_1)$ directly. The disintegration rate may be measured experimentally by means of a Geiger-Müller counter-tube. In its simplest form a Geiger-Müller tube is either a metal or glass cylinder with a coaxial wire of platinum or tungsten which forms the anode. The cylinder of metal tubes forms the cathode, while glass tubes either contain a cylinder of metal foil or are coated with colloidal graphite or evaporated metal films. A high potential difference is produced between anode and cathode. The tube is filled with an inert gas plus ethanol or ethyl formate vapour. When the radiation enters the counter it produces ionization and the discharge of the negative ions on the highly charged anode wire causes a pulse. The pulses are amplified and operate a mechanical register which records the number of charged particles entering the tube. By such measurements the radioactive isotopes used as tracers are detected and quantitatively assayed.

The counts obtained in a Geiger-Müller tube must be corrected for the *background count*, which is the count recorded when the tube is operated without any known radioactive source in position. Such counts are caused mainly by cosmic rays which produce ionizations in the counter tube.

Units and Definitions.

The *curie* (c.) is the amount of radioactive isotope necessary to produce the same number of nuclear disintegrations as 1 gram of radium, namely $3{\cdot}7 \times 10^{10}$ disintegrations per second. This amount is far too great for normal biological use, and microcurie (μc.) and millicurie (mc.) quantities, 10^{-6} and 10^{-3} curies respectively, find application as tracers.

Specific Activity is the ratio of the radioactive atoms of an element to the total atoms of the same element present in the mixture, e.g. $^{35}S/(^{32}S + {}^{35}S)$, and is usually expressed as curies, millicuries or microcuries per gram or milligram of element. Similar notation is used for compounds of the element, e.g. mc. of radioactive isotope per mg. of compound, although a better notation is mc. per millimole, which facilitates comparison with compounds of different molecular weight.

Radioactivity, when measured by Geiger-Müller counter, is usually expressed as counts per minute for a given weight of material. These counts are purely arbitrary units, and in biological work usually no effort is made to convert the count rates into millicuries. Commercially produced radioactive isotopes are supplied with the specific activity given in terms of μc. or mc. per ml. of solution or per mg. or g. of solid. Consequently the term specific activity is applied to two different types of measurement.

Relative Specific Activity.—Specific activities are frequently expressed relative to the specific activity of a reference compound, e.g. organic phosphates relative to inorganic phosphate of the tissue or of the blood; they are then termed relative specific activities.

Turnover.—This term is used to denote the renewal of a given substance by synthesis or by exchange or by entering of a labelled molecule into a tissue. Net synthesis can, of course, be studied by methods other than isotopic, but the tracer technique is particularly suited for demonstrating the incorporation of new molecules which is being balanced by removal of molecules already present, i.e. where there is no net increase in concentration and classical analytical methods are of no avail.

Turnover Rate.—The amount of the substance that is turned over by the tissue per unit time is the turnover rate of the substance.

Turnover Time.—This is the time required for the turnover of an amount of the substance equal to that present in the tissue.

Isotope Dilution.

A very valuable method of analysis has been placed at the disposal of the biochemist with the advent of tracer techniques. Many compounds are present in mixtures of biological substances (amino acids in protein hydrolysates, for example) in such small amount that quantitative isolation of the pure substance is extremely difficult or even impossible. However, if a pure labelled sample of the same compound is added to the biological material, it mixes with and becomes indistinguishable from the compound already present. If now a sample of the substance is isolated in pure form, regardless of yield, the specific radioactivity may be measured, and knowing the specific activity and weight

of the added material, it is possible to calculate the amount of unlabelled compound originally present in the mixture.

Suppose that a grams of labelled compound displaying n_1 counts per minute, and therefore having a specific activity of n_1/a, are added to a mixture containing b grams of the unlabelled compound. A pure sample of the compound is now isolated, say c grams, and found to give n_2 counts per minute. The specific activity is therefore n_2/c. Measurement of the specific activity before and after dilution permits b to be determined since

$$\frac{n_2}{c} = \frac{n_1}{(a+b)} \quad . \quad . \quad . \quad (11.1)$$

whence

$$b = \frac{n_1 c - n_2 a}{n_2} \quad . \quad . \quad . \quad (11.2)$$

and if the specific activities of added and isolated material are denoted by $s.a._1$ and $s.a._2$ respectively, then equation 11.2 becomes

$$b = a\left(\frac{s.a._1}{s.a._2} - 1\right) \quad . \quad . \quad . \quad (11.3)$$

With radioactive isotopes the difference in atomic weight between the radioactive and stable isotope, and hence between the molecular weight of labelled and unlabelled compound, becomes vanishingly small and may be neglected. This is not so with stable heavy isotopes, where the atom per cent. excess of labelling element used may be sufficient to justify taking it into consideration. In these cases equation 11.3 becomes

$$b = a\left(\frac{e_1}{e_2} - 1\right)\frac{M_2}{M_1} \quad . \quad . \quad . \quad (11.4)$$

where e_1 and e_2 are the atom per cent. excess of added and isolated material and M_1 and M_2 their molecular weights. When the difference in molecular weights is insignificant, M_2/M_1 in equation 11.4 becomes unity. Note that with stable isotopes it is not even necessary to know the weight of the isolated pure material because the atom per cent. excess determined by mass spectrometry is all that is required, whereas the weight is necessary for the determination of specific radioactivity. It is desirable that the added material should have an atom per cent. excess greater than 5 for isotope dilution analysis.

Example 11.1.—5·1 mg. of ^{14}C-labelled arginine giving 2,623 counts per minute were added to a protein hydrolysate and then a sample of arginine was isolated from the mixture and purified to yield 12·5 mg. The resultant material gave 1,047 counts per minute when assayed in a Geiger-Müller tube. If the counter had a background correction of 15 counts per minute, determine the amount of arginine present in the hydrolysate.

Applying the background correction, the true counts per minute are 2,608 and 1,032 for added and isolated materials respectively, and the specific activities are 2,608/5·1 and 1,032/12·5. Let x mg. be the amount of arginine in the hydrolysate then, using equation 11.3

$$x = 5{\cdot}1\left(\frac{2608 \times 12{\cdot}5}{5{\cdot}1 \times 1032} - 1\right) = 31{\cdot}6 - 5{\cdot}1 = \underline{26{\cdot}5 \text{ mg.}}$$

Identification of Precursors.

The isotopic tracer technique affords a method for the study of the conversion of a compound A into compound B or for the utilization of part of A in the synthesis of B. Labelled substance A is administered to the intact animal or bacterial suspension, or to tissue slices, homogenates or cell-free extracts, followed by analysis to discover whether labelling occurs in compound B. Such experiments are of qualitative nature since labelled A will be diluted with any non-labelled A present in the system or formed from other sources during the course of the experiment.

A mathematical approach to the precursor problem has been made by Zilversmit, Entenman and Fishler (1943) by considering the specific activities of precursor and product as a function of time. Certain assumptions are made. It is necessary to assume that the compound studied is in the steady state over the interval of time considered, i.e. its rate of appearance is equal to its rate of disappearance. Furthermore, the rates of appearance and disappearance of the compound must be constant over the time period used for the calculation, and there must be random appearance and disappearance of all molecules, i.e. the organism does not distinguish between 'old' and 'newly' formed molecules.

If compound A is the immediate precursor of B, and the labelled material administered is some compound other than A, the ideal relationship between them is shown in Fig. 11.1. It will be noticed that both A and B have zero specific activity at zero time and that when the specific activity of B has reached its maximum it is equal to that of the precursor A. Prior to this time the specific activity of A has reached its maximum, and after the intersection of the curves the specific activity of A is less than that of B.

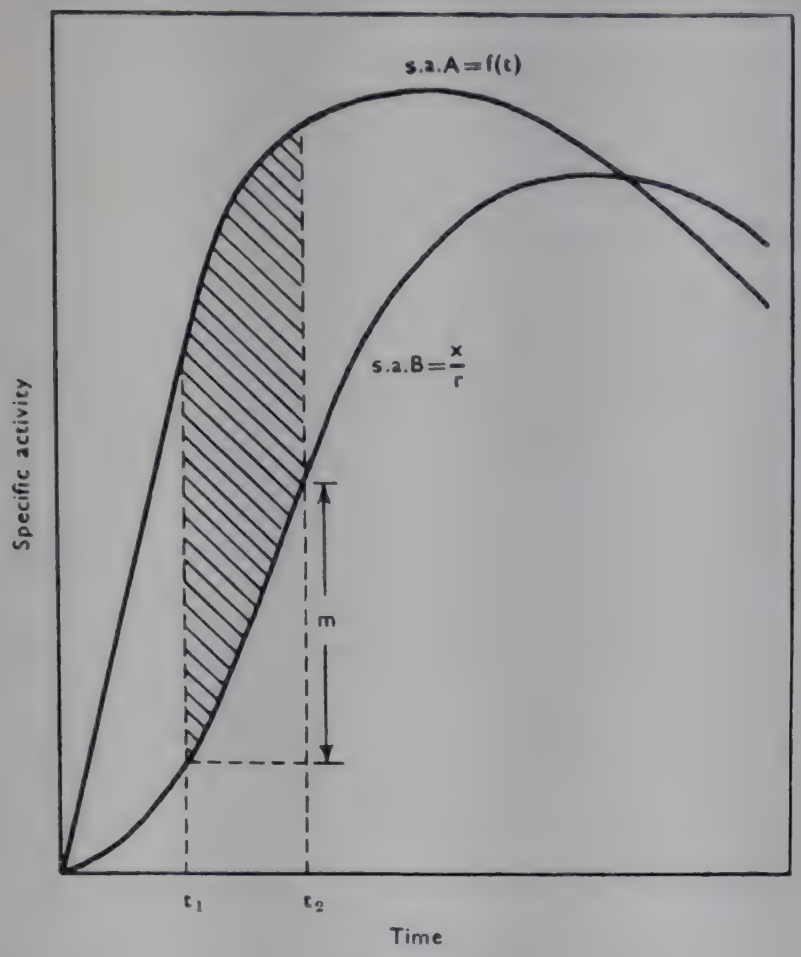

FIG. 11.1

Relationship between specific activity and time for compounds A and B, where A is the precursor of B and the labelled material administered is a compound other than A. (ZILVERSMIT, ENTENMAN & FISHLER, 1943.)

The mathematical expression derived by these workers is:

$$\frac{dx}{dt} = pf(t) - p\frac{x}{r} \quad . \quad . \quad . \quad (11.5)$$

where p = the rate of conversion of A to B which is assumed to be constant

r = the amount of B present in the tissue, also assumed to be constant, i.e. in the steady state

x = the amount of radioactive B present in the tissue and

$f(t)$ = the specific radioactivity of the immediate precursor A, expressed as a function of time.

Since r is constant,

$$r\left(\frac{d\frac{x}{r}}{dt}\right) = p\left[f(t) - \frac{x}{r}\right] \quad . \quad . \quad (11.6)$$

which becomes

$$\frac{\left(\frac{d\overset{x}{r}}{dt}\right)}{f(t)-\frac{x}{r}}=\frac{p}{r}=\text{constant} \quad . \quad . \quad . \quad (11.7)$$

The numerator of the left-hand side of equation 11.7 measures the slope of the specific activity-time curve for B and the denominator is the difference between the specific activities of A and B at any time t. The right-hand side of the equation is constant so that the slope of the specific activity-time curve for B is proportional to the difference between the specific activities of precursor and product.

Unfortunately it is not always possible to obtain all the numerical values required by this approach on a single animal. Where it can be done, the turnover time of B is given by the ratio of the area between the two specific activity curves to the slope of the curve for the specific activity of B, over any time period, i.e. it may be in either the rising or falling portions of the curve. In Fig. 11.1 the turnover time is given by the area of the shaded portion of the curve divided by m, which is the difference in specific activity of B over the time interval $t_2 - t_1$. Knowledge of the amount of B present in the tissue is not necessary for the calculation of the turnover time but permits determination of the turnover rate, which may be expressed as μg. or mg. of B turned over per gram of tissue per hour. The turnover time is equal to r/p.

In the majority of experiments it does not prove possible to obtain all the required data from a single animal and it becomes necessary to compare relative specific activities for the animals employed.

Limitations and Precautions.

Reaction Rates of Isotopes.—Although isotopes are almost identical in chemical properties there are some slight differences which must be borne in mind when using isotopes as tracers. For instance, there are slight differences in reaction rates: although isotopes undergo the same reactions, a heavy isotope reacts more slowly than a lighter one. The most pronounced

example is deuterium, which has a mass double that of protium (hydrogen, mass 1) and which may, under the most unfavourable conditions, react at one-eighteenth of the rate of protium. Where the labelling atom is not directly involved in the reaction, as for example when it maintains its position in the molecule throughout the reactions followed, there is no effect on the reaction rate.

Exchange Reactions.—Care must be taken to ensure that the labelled atom occupies a position in the molecule where it will not undergo spontaneous exchange with unlabelled atoms present in the medium. This consideration excludes the use of deuterium in carboxyl, hydroxyl and amino groups because rapid exchange occurs between the deuterium and the hydrogen of the aqueous medium. Many other types of exchange reaction are known. To quote an example, deuterium-labelled valine may be prepared with the isotope in the β and γ positions by means of an exchange reaction with D_2SO_4.

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PROBLEMS

11.1. ^{32}P has a half-life of 14·3 days. Calculate the decay constant and determine what percentage of the initial radioactivity remains after 10, 20, 30 and 50 days.

11.2. In an experiment with ^{32}P (half-life = 14·3 days) a sample containing 50 μg. phosphorus was assayed in a Geiger-Müller counter with a background count of 14 counts per minute. The recorded count was 2,640 in 10 minutes. If the sample was assayed 14·3 days after commencement of the experiment what was the specific activity of the phosphorus (in counts/min./100 μg. total phosphorus) at the start of the experiment?

(Glasgow Double Science Course, 1952)

11.3. Radioactive sulphur ^{35}S was assayed over a period of 15 days and the following counts recorded in a Geiger-Müller counter. Determine the half-life period and the decay constant for this isotope.

Time (days)	0	1	2	3	4	5	10	15
Counts/min.	4280	4245	4212	4179	4146	4113	3952	3798

11.4. Shemin and Rittenberg demonstrated that glycine is a specific precursor of the haem present in red blood cells. Glycine labelled with ^{15}N was

ingested over a period of 3 days by the subject (Shemin) and the isotope content of haemoglobin isolated from red blood cells determined during the course of several months. The following data were obtained:

Time (days)	0	4	18	77	86	99
^{15}N atom per cent. excess	0·000	0·134	0·422	0·466	0·462	0·448
Time (days)	127	134	154	170	192	231
^{15}N atom per cent. excess	0·342	0·200	0·164	0·112	0·096	0·062

Express these data graphically. What explanation can you offer for the observed behaviour of the isotope content of the haem?

(After SHEMIN & RITTENBERG (1946), *J. biol. Chem.*, **166**, 627.)

11.5. 3·0 grams of dry haemoglobin were hydrolysed and racemized by treatment with 33 per cent. sulphuric acid in a sealed ampoule at 170°. The hydrolysate was mixed with 0·392 g. DL-leucine containing deuterium. The sulphuric acid was then removed and the amino acid mixture dried and powdered. The residue was extracted to remove unwanted material, and the leucine isolated as the copper salt and purified by recrystallization from water.

The excess density of water formed by combustion of the heavy leucine was 518 parts per million and that of the leucine isolated from the hydrolysate 161 parts per million.

Calculate the weight of leucine present in the hydrolysate and hence the leucine content of haemoglobin as a percentage.

(After USSING (1939), *Nature*, **144**, 977.)

11.6. An experiment was carried out in order to study the biosynthesis of phospholipids in the laying hen. Radioactive inorganic phosphate was administered intravenously at frequent intervals so as to maintain a constant blood level of inorganic ^{32}P over a period of 72 hours. At the end of this time the animal was sacrificed and the phospholipids of the liver, blood and yolks were isolated for analysis. The following results were obtained:

Substance	*P in sample counted* (μg.)	*Counts/minute*
Blood lecithin .	23·2	474
Blood cephalin .	14·4	495
Liver lecithin .	17·4	360
Liver cephalin .	12·0	246
Yolk lecithin .	24·0	1440
Yolk cephalin .	19·2	379

What conclusions can you draw as to the biosynthesis of liver, blood and yolk phospholipids?

(Glasgow Double Science Course, June 1954.)

11.7. The palmitic acid content of a mixture of fatty acids derived from rat fat has been determined by the isotope dilution method. Deuteriopalmitic acid was added to the mixture and then a sample of pure palmitic acid isolated and its isotope content determined by mass spectrometer. Results from two experiments are recorded below.

Total fatty acids in mixture g.	14·641	14·135
Labelled palmitic acid added g.	0·2163	0·1757
Deuterium content of added palmitic acid, per cent. .	21·5	21·5
Deuterium content of isolated palmitic acid, per cent. .	1·28	1·18

Calculate the amount of palmitic acid present from the figures obtained in each experiment. The difference in molecular weight between palmitic and deuteriopalmitic acids may be neglected.

(After RITTENBERG & FOSTER (1940), *J. biol. Chem.*, **133**, 737.)

11.8. Amino acid analyses of horse haemoglobin have been carried out by Foster using the isotope dilution technique. A weighed sample of the protein was hydrolysed for 18-20 hours with 6 N HCl, followed by addition of a weighed amount of isotopic DL-amino acid of known ^{15}N excess to the hydrolysate. Isolation of the natural (L) isomer of the amino acid in a high state of purity was achieved and the atom per cent. ^{15}N excess determined by the mass spectrograph. The following data were obtained:

Amino acid added			*Mean ^{15}N excess in compound isolated*	*Weight of protein hydrolysed*
Compound	*Amount of L-amino acid* g.	*^{15}N excess* atom per cent.	atom per cent.	g.
Tyrosine . .	0·0740	6·85	0·699	21·65
Phenylalanine .	0·1296	6·79	0·472	25·60
Arginine . .	0·0850	8·72	0·838	21·60
Lysine . .	0·1372	9·09	0·558	24·58
Glutamic acid .	0·2030	4·52	0·383	25·60
Aspartic acid .	0·1642	6·75	0·450	22·13
Glycine . .	1·1869	1·15	0·570	22·13
Leucine . .	0·0767	6·73	0·638	4·85

Calculate the percentage composition of haemoglobin with respect to the analysed amino acids and, assuming the molecular weight of the protein to be 66,700, determine the number of residues of each amino acid per mole of haemoglobin.

(After FOSTER (1945), *J. biol. Chem.*, **159**, 431.)

11.9. Give an account of the mechanism of urea formation in the mammalian organism.

α-^{15}N-Ornithine or δ-^{15}N-ornithine was fed over several days to a group of mice and the amino acids of the tissue proteins then assayed for ^{15}N. Some of the results obtained are given in the following table:

Contributions of α- and δ-nitrogen atoms of ornithine to some amino acids

Amino acid	*N derived from δ-N of ornithine* per cent.	*N derived from α-N of ornithine* per cent.
Arginine		
Amidine N .	0·56	0·42
α-Amino N .	0·02	6·48
δ-Amino N .	6·24	0·00
Glutamic acid .		0·75
Aspartic acid .		0·56
Proline . .		1·41
Hydroxyproline .		0·46

Discuss the significance of these results.

(After STETTEN (1951), *J. biol. Chem.*, **189**, 499; Glasgow Double Science Course, March 1954.)

11.10. Amino acid analyses of β-lactoglobulin have been carried out by Foster using the same experimental techniques as for haemoglobin (Problem 11.8).

Data obtained in isotope dilution experiments are recorded below. From this information express the amino acid contents as percentages and also determine the number of amino acid residues per mole of β-lactoglobulin, the molecular weight of which may be assumed to be 42,000.

Amino acid added			*Mean ^{15}N excess in compound isolated*	*Weight of protein hydrolysed*
Compound	*Amount of L-amino acid* g.	^{15}N *excess* atom per cent.	atom per cent.	g.
Glutamic acid .	0·1484	4·62	0·448	7·05
Aspartic acid .	0·0681	6·75	0·534	7·05
Lysine . .	0·0660	9·09	0·686	7·05
Leucine . .	0·0688	6·73	0·396	7·05
Glycine . .	0·3147	1·99	1·485	7·05

(After FOSTER (1945), *J. biol. Chem.*, **159**, 431.)

11.11. Experiments to discover the immediate phosphorus precursor of phospholipins (lecithin and cephalin) have been carried out by Popjak and Muir.

Rats in groups of four to six were injected subcutaneously with Na_2HPO_4 labelled with ^{32}P. The members of the group were killed at different intervals after the injection, the liver excised, frozen and ground to a powder. The powdered liver was extracted and the extract fractionated for acid-soluble phosphates. Five fractions were obtained corresponding to inorganic phosphate, ?phosphoglyceric acid, α- and β-glycerophosphates, adenylic acid and phospholipin, and each was assayed in a Geiger-Müller counter. Results obtained are recorded below.

Use these data to deduce the possible immediate precursor of liver phospholipins and also the turnover time of the phospholipins.

Time after injection of ^{32}P (min.)	*Fraction A* *Inorganic P*	*Fraction B* *?Residual inorganic P* *?Phosphoglyceric acid*	*Fraction C* *α- and β-glycerophosphate*	*Fraction D* *Adenylic acid*	*Fraction E* *Phospholipin P*
50	4·4	2·80	2·65	1·20	0·15
60	5·2				
100	5·1	3·80	2·45	1·85	0·30
182	3·55		2·45		
190	3·38		2·20		
240					0·70
250	3·02		2·10	1·95	
255		2·85			
440	2·22		1·70	1·90	1·35
530	2·05	1·90	1·60	1·75	1·30
685			1·48		1·48
875	1·40		1·20	1·40	1·35
1325	1·10	0·90	0·95	1·10	1·03

(After POPJAK & MUIR (1950), *Biochem. J.*, **46**, 103.)

11.12. *Torulopsis utilis* is grown on (1—^{14}C) glucose as sole carbon source. After harvesting, phenylalanine is isolated from a protein fraction and degraded (*a*) by treatment with ninhydrin to yield CO_2 and (*b*) by permanganate oxidation to yield benzoic acid. The benzoic acid is decarboxylated to give benzene.

All compounds studied are combusted to CO_2 prior to counting and all specific activities in the following table are given as counts per min. per μmole of CO_2 of combustion.

Compound studied	*Specific activity of CO_2 of combustion*
(1—^{14}C) glucose in medium	340
phenylalanine	220
CO_2 (ninhydrin)	10
benzoic acid	280
benzene	170

Calculate the specific activities of each of the side chain carbon atoms and suggest a possible origin of this three carbon fragment.

(Glasgow Double Science Course, June 1955.)

11.13. The amino acid composition of crystalline human serum albumin has been determined by the isotope dilution technique. After hydrolysis of a weighed amount of the protein, ^{15}N-labelled amino acids were added to the hydrolysate and, after mixing, either metal or other derivatives of the amino acids isolated and purified. The data obtained are tabulated below.

Amino acid added			*Mean ^{15}N excess in compound isolated*	*Weight of protein hydrolysed*
Compound	*Amount of L-amino acid* mg.	^{15}N *excess* atom per cent.	atom per cent.	g.
Glutamic acid .	89·37	18·69	1·187	7·80
Aspartic acid .	91·05	11·82	1·269	7·80
Tyrosine . .	77·65	6·85	1·159	7·98

Calculate the percentage of each amino acid present and also determine the number of residues of each amino acid per molecule of human serum albumin of molecular weight 70,000.

(After SHEMIN (1945), *J. biol. Chem.*, **159**, 439.)

Appendix 1

SYMBOLS

A	area, absorption ratio
a_i	activity of ion i
C	concentration
c	concentration, velocity of light
D	diffusion coefficient
E	activation energy, electrode potential, extinction (optical density)
F	Faraday
F	molar frictional coefficient
f	molecular frictional coefficient
f_i	activity coefficient of ion i
G	free energy
H	heat content (enthalpy)
h	Planck's constant ($6{\cdot}62 \times 10^{-27}$erg sec.)
I	ionic strength, light intensity
K	equilibrium constant, extinction coefficient
K_a	thermodynamic equilibrium constant (activities)
K_a	acidic dissociation constant
$K_{app.}$	apparent dissociation constant
K_b	basic dissociation constant
K_c	equilibrium constant (concentrations)
K_i	enzyme-inhibitor dissociation constant
K_s	Michaelis constant
k	rate constant, Boltzmann's constant ($1{\cdot}37 \times 10^{-16}$erg/deg.)
l	length
M	moles per litre (molar)
M	molecular weight
M_n	number-average molecular weight
M_w	weight-average molecular weight
m	milli (10^{-3})
N	Avogadro's number ($6{\cdot}06 \times 10^{23}$)
N	normality (gram-equivalents per litre)
P	pressure, steric factor
pf_i	$-\log f_i$

pH	$-\log (H^+)$
pI	isoelectric point
pK_a	$-\log K_a$
Q	heat absorbed, Q_v at constant volume, Q_p at constant pressure
Q_x	metabolic quotient with substrate
Q_{10}	temperature coefficient
R	gas constant
R.Q.	respiratory quotient
r	radius
rH	$-\log (H_2)$
S	entropy, Svedberg unit (10^{-13} sec.)
s	sedimentation constant
T	absolute temperature
t	time
$t_{\frac{1}{2}}$	half-life period
U	internal energy
V	volume, velocity of reaction
v	volume
$\bar{v}$	partial specific volume
W	work done by system
Z	molecular collision rate
z	valency
α	degree of dissociation, solubility coefficient, angle of rotation
β	buffer value
Δ	measurable increment
δ	infinitesimal increment
ϵ	molar extinction coefficient
$\epsilon_{spec.}$	specific extinction coefficient
η	viscosity coefficient
$[\eta]$	intrinsic viscosity
η_r	relative viscosity coefficient
$\eta_{sp.}$	specific viscosity
λ	wavelength
μ	micro (10^{-6})
ν	frequency
Π	osmotic pressure
ρ	density

Σ	sum
ϕ	volume fraction
ω	angular velocity
$^\circ$	standard state
$\ddagger$	transition state (activated complex)
( )	activity
[ ]	concentration

Appendix 2

THE GAS CONSTANT

The universal gas equation is expressed by the relationship

$$PV = RT$$

and the value of R obviously depends on the units used to express P and V.

Where P is expressed in atmospheres and V in litres, at N.T.P. $R = \frac{1 \times 22{\cdot}4}{273}$ = 0·08204 litre atmospheres per mole per degree.

However, if P is expressed in dynes per cm.^{2} and V in ml.

$$P = 76 \times 13{\cdot}59 \times 981 \text{ dynes/cm.}^2$$

and $$R = \frac{76 \times 13{\cdot}59 \times 981 \times 22400}{273} = 8{\cdot}314 \times 10^7 \text{ ergs/mole/degree}$$

$$= 8{\cdot}314 \text{ joules/mole/degree.}$$

Furthermore, since $4{\cdot}184 \times 10^7$ ergs = 1 calorie

$$R = \frac{8{\cdot}314}{4{\cdot}184} = 1{\cdot}987 \text{ calories per mole per degree.}$$

Quite often in osmotic pressure measurements the pressure is determined in cm. of water instead of mercury, since this means a larger value to read experimentally. In these cases

$$R = \frac{76 \times 13{\cdot}59 \times 22400}{273} = 8{\cdot}471 \times 10^4 \text{ ml. cm. } H_2O\text{/mole/degree.}$$

Appendix 3

THE GRAPHICAL SOLUTION OF PROBLEMS

Many of the problems in this book demand graphical solution, and a few notes on this topic may be of value.

Choice of scale is very important, especially where the slope of the line is required. The scale should be chosen so that the angle of the slope is approximately 45°, since this offers maximum accuracy. The scale selected should be large enough for the error in setting out the points to be small in comparison with the random measuring error, i.e. the spread of the points on either side of the correct line.

The slope of a straight line can be found by drawing the best possible straight line through the experimental points. The slope of this best line may be checked from the experimental points x_1y_1, x_2y_2 . . . etc. by the formula

$$\frac{(y_1 + y_2 + \ldots + y_n) - (y_{n+1} + y_{n+2} + \ldots + y_{2n})}{(x_1 + x_2 + \ldots + x_n) - (x_{n+1} + x_{n+2} + \ldots + x_{2n})}.$$

Appendix 4

ANGULAR VELOCITY

The calculation of molecular weights from sedimentation data demands a knowledge of the angular velocity of the centrifuge in radians per second. If the angle described in time t is θ, the angular velocity ω is given by

$$\omega = \frac{\theta}{t}$$

and where θ is in radians and t in seconds ω is obtained in radians per second.

Since a radian is the angle subtended by an arc equal to the radius of a circle, the circumference of which is $2\pi r$, it follows that one circumference or revolution is equal to 2π radians. Hence multiplication of the speed of the centrifuge in revolutions per second by 2π gives the angular velocity in radians per second.

Appendix 5

INTERNATIONAL ATOMIC WEIGHTS

	Symbol	*Atomic Number*	*Atomic Weight*
Aluminium	Al	13	26·97
Antimony	Sb	51	121·76
Argon	A	18	39·944
Arsenic	As	33	74·91
Barium	Ba	56	137·36
Beryllium	Be	4	9·02
Bismuth	Bi	83	209·00
Boron	B	5	10·82
Bromine	Br	35	79·916
Cadmium	Cd	48	112·41
Caesium	Cs	55	132·91
Calcium	Ca	20	40·08
Carbon	C	6	12·01
Cerium	Ce	58	140·13
Chlorine	Cl	17	35·457
Chromium	Cr	24	52·01
Cobalt	Co	27	58·94
Copper	Cu	29	63·57
Dysprosium	Dy	66	162·46
Erbium	Er	68	167·2
Europium	Eu	63	152·0
Fluorine	F	9	19·00
Gadolinium	Gd	64	156·9
Gallium	Ga	31	69·72
Germanium	Ge	32	72·60
Gold	Au	79	197·2
Hafnium	Hf	72	178·6
Helium	He	2	4·003
Holmium	Ho	67	164·94
Hydrogen	H	1	1·0081
Indium	In	49	114·76
Iodine	I	53	126·92
Iridium	Ir	77	193·1

	Symbol	*Atomic Number*	*Atomic Weight*
Iron	Fe	26	55·84
Krypton	Kr	36	83·7
Lanthanum	La	57	138·92
Lead	Pb	82	207·21
Lithium	Li	3	6·940
Lutecium	Lu	71	175·00
Magnesium	Mg	12	24·32
Manganese	Mn	25	54·93
Mercury	Hg	80	200·61
Molybdenum	Mo	42	95·95
Neodymium	Nd	60	144·27
Neon	Ne	10	20·183
Nickel	Ni	28	58·69
Niobium	Nb	41	92·91
Nitrogen	N	7	14·008
Osmium	Os	76	190·2
Oxygen	O	8	16·000
Palladium	Pd	46	106·7
Phosphorus	P	15	30·98
Platinum	Pt	78	195·23
Potassium	K	19	39·096
Praseodymium	Pr	59	140·92
Protactinium	Pa	91	231
Radium	Ra	88	226·05
Radon	Rn	86	222
Rhenium	Re	75	186·31
Rhodium	Rh	45	102·91
Rubidium	Rb	37	85·48
Ruthenium	Ru	44	101·7
Samarium	Sm	62	150·43
Scandium	Sc	21	45·10
Selenium	Se	34	78·96
Silicon	Si	14	28·06
Silver	Ag	47	107·880
Sodium	Na	11	22·997
Strontium	Sr	38	87·63
Sulphur	S	16	32·06
Tantalum	Ta	73	180·88
Tellurium	Te	52	127·61

	Symbol	*Atomic Number*	*Atomic Weight*
Terbium . .	Tb	65	159·2
Thallium . .	Tl	81	204·39
Thorium . .	Th	90	232·12
Thulium . .	Tm	69	169·4
Tin . . .	Sn	50	118·70
Titanium . .	Ti	22	47·90
Tungsten . .	W	74	183·92
Uranium . .	U	92	238·07
Vanadium .	V	23	50·95
Xenon . .	Xe	54	131·3
Ytterbium . .	Yb	70	173·04
Yttrium . .	Y	39	88·92
Zinc . . .	Zn	30	65·38
Zirconium . .	Zr	40	91·22

ANSWERS

FOUR or five figure logarithmic tables have been used, where occasion demands, in the solution of the problems.

Where a problem requires graphical solution there is, of course, the possibility of deviation from the recorded answer due to personal assessment of the best straight line through the experimental points. The answer recorded in such cases is, wherever possible, the mean of two individual determinations. Furthermore, it is possible to solve some problems by alternative methods and the answers do not always correspond exactly.

CHAPTER I

1.1. 600; 2S, 3P.
1.2. Horse: Fe 16,669; S-S 16,878; S 16,446. 1 Fe, 1 S-S, 2S. Pig: Fe 13,960; S 13,362. 1 Fe, 2 S.
1.3. Tryptophan 36,447, 3 mol.; tyrosine 36,216, 9 mol.; β-hydroxyglutamic acid 36,244, 4 mol.
1.4. 19,866; 18,697; 16,729; 21,920; 36,700.
1.5. Fe 33,238; S 33,405; arginine 32,856. 2 Fe, 5 S, 8 arginine.
1.6. 1 Fe combines with 0·944 $\approx$ 1 mole oxygen.
1.7. 750; 780.
1.8. 1,090; 1,430.
1.9. 49; 47.
1.10. 412.
1.11. 690.
1.12. Acid 1,047, acidic dye 961·3; base 1,429, basic dye 1,429.
1.13. M_n 46,750; M_w 51,612.
1.14. 2·629 atm.
1.15. 59·98 $\approx$ 60.
1.16. 62,320.
1.18. A 59,990; B 56,990; greater solvation effect with B.
1.20. Mean value 74,600.
1.21. ca. 6·7.
1.22. 69,900.
1.23. 66,800.
1.24. 66,700.
1.25. 2·08 days, 13,600; 10·2 days, 6,100.
1.26. Mean value 5,930; individual values, 5,900, 5,960, 5,930, 5,930.
1.27. 1,475,000; 1,060,000; 1,291,000; 1,235,000.
1.28. 184,000 before, 98,000 after treatment.
1.29. 34,800.
1.30. 61,500.
1.31. 270,000.
1.32. 35,210; 60,970 cm.^{3}
1.33. By activity: 0·0214, 0·0217, 0·0220; by nitrogen: ——, 0·0212, 0·0228 cm.^{2}/day. Average of all determinations 0·02185 giving $r = 2{\cdot}64 \times 10^{-7}$ cm. and $V = 46{,}710$ cm.^{3}
1.34. Volume of water of hydration per g. dry trypsin: 0·50, 0·49, 0·50, 0·55, 0·74 ml. (in order of increasing trypsin concentration).
1.35. Reduced osmotic pressure 60,000; Kunitz average 46,870.
1.36. Saccharose: 0·00578, 0·0104; 0·0120, 0·0216; 0·0313, 0·0564; 0·0731, 0·132; 0·127, 0·228; 0·204, 0·367. Glucose: 0·0138, 0·0248; 0·0289, 0·0520; 0·0702, 0·126; 0·138, 0·248; 0·200, 0·360; 0·270, 0·486 (Kunitz value given first in each case; values in order of increasing sugar concentration).

1.37. Volume of water of hydration per g. HbCO: 0·14, 0·18, 0·27, 0·43, 0·55 ml., in order of increasing HbCO concentration. The effect of concentration on the degree of hydration is much less pronounced if the Kunitz equation is used in its full form. The corresponding values are then 0·13, 0·12, 0·14, 0·20 and 0·22 ml./g. HbCO.
1.38. 180.
1.39. 73,000.

CHAPTER II

2.1. 1×10^{-6}; $3{\cdot}16 \times 10^{-10}$ g./l.
2.2. 0·869.
2.3. (*a*) 3. (*b*) 11; 3·95.
2.4. $1{\cdot}93 \times 10^{-2}$ M.
2.5. (*a*) 0·1; (*b*) 3; (*c*) 0·1; (*d*) 0·12; (*e*) 0·45; (*f*) 3; (*g*) 0·075.
(1) I = M; (2) I = 3M; (3) I = 4M; (4) I = 6M; (5) I = 15M.
2.6. $4{\cdot}24 \times 10^{-4}$.
2.7. (*a*) f_{H^+} 0·869; a_{H^+} $8{\cdot}69 \times 10^{-3}$; $f_{SO_4''}$ 0·569; $a_{SO_4''}$ $2{\cdot}85 \times 10^{-3}$.
(*b*) $f_{Na^+} = f_{Cl'} = 0{\cdot}91$; $a_{Na^+} = a_{Cl'} = 1{\cdot}82 \times 10^{-3}$.
2.8. 0·025; $f_{Mg^{++}}$ 0·483; $a_{Mg^{++}}$ $3{\cdot}62 \times 10^{-3}$; $f_{Cl'}$ 0·834; $a_{Cl'}$ $8{\cdot}34 \times 10^{-3}$; $f_{SO_4''}$ 0·483; $a_{SO_4''}$ $1{\cdot}21 \times 10^{-3}$.
2.9. $[H_2CO_3]/[HCO_3'] = 0{\cdot}082/1{\cdot}0$.
2.10. $1{\cdot}61 \times 10^{-7}$.
2.11. Oxyhaemoglobin. [acid]/[salt]: haemoglobin 6·17/1·0; oxyhaemoglobin 0·17/1·0.
2.12. (*a*) 6·00; (*b*) 6·90; (*c*) 5·41; (*d*) 5·05; (*e*) 6·02; (*f*) 6·12; (*g*) 5·65; (*h*) 5·12.
2.13. (*a*) 5·60; (*b*) 5·60; (*c*) 3·05; (*d*) 7·30; (*e*) 5·70; (*f*) 5·69; (*g*) 3·31.
2.14. β 0·079. Maximum buffering range *ca.* pH 3·0-5·70.
2.15. pK_{a_2} 6·82; |pH, β|6·82, 0·55|6·0, 0·23|7·5, 0·28|.
2.16. 7·41.
2.17. Arterial: p_{CO_2} 38·3 mm.; $[CO_2]$ 1·15 mM; $[HCO_3']$ 25·24 mM.
Venous: p_{CO_2} 47·1 mm.; $[CO_2]$ 1·81 mM; $[HCO_3']$ 25·92 mM.
2.18. Oxygenated: 12·5 mM; non-oxygenated: 26·6 mM.
2.19. Oxygenated: 19·85 mM; non-oxygenated: 28·96 mM.
2.20. Between pH 6·1 and 9·0 oxyhaemoglobin is the stronger acid; between pH 4·5 and 6·1 haemoglobin is stronger. Above pH 9 and below pH 4·5 there is no difference.
2.21. 22:1; 11:1; 2·82:1; 1·83:1; 1·08:1.
2.22. H_2CO_3: 1·997, 5·07, 4·87, 4·61, 4·44, 4·28 mM. pK_{a_1}': 6·267, 6·204, 6·177, 6·155, 6·134, 6·100. $pK_{a_1}' = -0{\cdot}53\sqrt{I} + 6{\cdot}327$; $\log f_{HCO_3'} = -0{\cdot}53\sqrt{I}$.
2.23. Compartment A: | Na^+, Cl' | 0·508, 0·498 | 1·333, 0·333 | 1·125, 0·125 | 1·000098, 0·000098 |. Compartment B: $Na^+ = Cl'$, 0·502, 0·667, 0·375, 0·0099. All concentrations mM.
2.24. Average value 6·60. Result probably rather high since limiting solubility imposes very low concentration of sulphadiazine for the experiment.

CHAPTER III

3.1. −2,380·0 kcal.
3·2. −2,693·4 kcal.
3.3. −325·13 kcal.; −63·31 kcal.
3.4. −691,040 cal.
3.5. −144,620 cal.
3.6. −3,350 cal.
3.7. 1·50 kcal.; 28·7%, 0·05%. This is rather an extreme case, but serves to emphasize the inaccuracy of the method.
3.8. −57·9 kcal.; −10·3 kcal.

3.9. −97·4 kcal.
3.10. −2,854 cal.
3.11. (*a*) 48·3; (*b*) 60·2; (*c*) 85·8; (*d*) 76·6; (*e*) 56·8.
3.12. 15,000 cal. energy bound in phosphoglycerylphosphate molecule.

CHAPTER IV

4.1. 807·2.
4.2. −78,594 cal.
4.3. −111,447 cal.
4.4. (*a*) 4,887; (*b*) 37·15; (*c*) 3·13.
4.5. $6·76 \times 10^{12}$; $3·85 \times 10^{-5}$ M; $7·94 \times 10^{16}$; $1·45 \times 10^{-8}$ M.
4.6. 17·52; −1770 cal.
4.7. 96·2% dihydroxyacetone phosphate; 3·8% glyceraldehyde phosphate.
4.8. −3,720 cal.
4.9. −3,800 cal./mole; 25°: −826 cal./mole; 38°: −703 cal./mole.
4.10. K_c (M × 10^{12}): (*a*) 7·23; (*b*) 7·46; (*c*) 7·76. Average: 7·48; ΔG°, +16,540 cal.
4.11. Reaction (3) $K_{app.}$ (litres/mole): 0·227, 0·190, 0·195, 0·166. Average: 0·195. Reaction (1) $K_{app.}$ (litres/mole): $8·37 \times 10^{3}$; ΔG°, −5295 cal.
4.12. $K_{app.}$ (litres/mole): 18·6, 17·7, 19·6, 21·5, 23·5. Average: 20·2. K_a(× 10^{-8}): 3·71, 3·53, 3·91, 4·29, 4·69. Average: 4·03. $K_{app.}$ (pyruvate-oxaloacetate): $4·71 \times 10^{-4}$.
4.13. pH 8·5: 0·0261, 0·0261, 0·0297, 0·0348. Average: 0·0292 M. ΔG°, 2,185 cal.; pH 5·8: 0·0122 M. ΔG°, 2,725 cal.
4.14. 298·1°: *K* 1·334, ΔG° −171 cal.; 310·7°: *K* 0·921, ΔG° +51 cal.; ΔH −5,405 cal.
4.15. Iso-citric/aconitic: 25°, 2·14; 38°, 1·54; citric/aconitic: 25°, 31·35; 38°, 20·72.
4.16. *K* values: 30 min. 6·81; 40 min. 6·78; 50 min. 6·82; equilibrium attained within 30 min.
4.17. [α-ketoglutarate] − [alanine] = 237 μl./ml. solution, and [glutamate] = [pyruvate] = 193 μl./ml. solution.
4.18. pH 6·5: 1·33, 1·52; pH 6·0: 1·36, 1·60; pH 7·5: 1·25, 1·57.
4.19. (*a*) 6·26, 6·14, 6·27, $6·13 \times 10^{-3}$, average value $6·20 \times 10^{-3}$. (*b*) Not necessarily, but this form of mass law equation is probably correct in general e.g. first power of all species. Could be $Prot' + Ca^{2-} \rightleftharpoons CaProt^{-}$, but not $2\ Prot'' - Ca^{2+}$. (*c*) Equilibrium not attained. Final concentrations: Ca^{2+}, 2·10; CaProt, 2·37; Prot″, 6·99 m-moles/kg. H_2O. (*d*) Not instantaneously in equilibrium; CaProt will dissociate. (*e*) Ca^{2+}, $1·39 \times 10^{-3}$; CaProt, $1·72 \times 10^{-3}$ m-moles kg. H_2O.

CHAPTER V

5.1. $t_{\frac{1}{2}}$, 45·1 days; *k*, 0·0154 $days^{-1}$.
5.2. First order.
5.3. Zero order.
5.4. (*a*) 8,100 cal./mole; (*b*) 12,200 cal./mole.
5.5. 18,200 cal./mole.
5.6. −67,700 cal.; +213 e.u.
5.7. Cat, 9,869 cal.; human, 10,140 cal.
5.8. 13,460 cal.
5.9. 12,000 cal.
5.10. 8,900 cal.
5.11. 9,139 cal.
5.12. First order; *k*, 0·055 $min.^{-1}$
5.13. Both first order. Oxaloacetate: *k*, 0·00140 $min.^{-1}$; oxalosuccinate: *k*, 0·00117 $min.^{-1}$
5.14. 70 kcal./mole. (From rate constants: 35·2, $5·63 \times 10^{-4}$; 30·2, $0·925 \times 10^{-4}$; 25·0, $0·13 \times 10^{-4}$ $sec.^{-1}$.)

5.15. First order, $k = 14{\cdot}2 \times 10^{-6}$ sec.$^{-1}$ Bimolecular; carry out reaction in dioxane containing low concentrations of water to demonstrate dependence on $[H_2O]$. $E = 21{,}700$ cal./mole; $\Delta G^{\ddagger} = 25{,}900$ cal. mole; $\Delta H^{\ddagger} = 21{,}100$ cal./mole; $\Delta S^{\ddagger} = -15{\cdot}3$ e.u.
5.16. $E = 12{,}000$ cal./mole. $k(26°) = 13{\cdot}2$ flashes/min.

CHAPTER VI

6.1. $5{\cdot}62 \times 10^{-3}$ M.
6.2. Serine: $2{\cdot}5 \times 10^{-3}$ M; threonine: $2{\cdot}2 \times 10^{-3}$ M.
6.3. pH 7·6.
6.4. $2{\cdot}3 \times 10^{-6}$ M.
6.5. $4{\cdot}1 \times 10^{-5}$ M.
6.6. Oxidation $\approx 10^{-5}$ M; for phosphorylation, concentrations are too far from K_S to give reliable values; best value is obtained with highest sarcosome concentration and is $\approx 5 \times 10^{-5}$ M.
6.7. $7{\cdot}81 \times 10^{-2}$ M; 0·1250 moles/hour.
6.8. 15°: $1{\cdot}01 \times 10^{-6}$ M; 22°: $1{\cdot}61 \times 10^{-6}$ M.
6.9. K_S $1{\cdot}25 \times 10^{-2}$ M; K_i $9{\cdot}92 \times 10^{-3}$ M.
6.10. Hydrocinnamate, phenylacetate and phenylbutyrate competitive; benzoate non-competitive.
6.11. 2,565,000.
6.12. Competitive; $6{\cdot}08 \times 10^{-6}$ M.
6.13. $6{\cdot}0 \times 10^{-5}$ M.
6.14. B non-competitive; C competitive.
6.15. $1{\cdot}66 \times 10^{-5}$ M.
6.16. $K_S = 1{\cdot}9 \times 10^{-3}$ M (rejecting 0·32 mM Mg^{2+} value); competitive; average $K_i = 5{\cdot}5 \times 10^{-4}$ M (rejecting 0·32 mM Mg^{2+} experiment.)

CHAPTER VII

7.1. 4·01, 2·46, 2·31.
7.2. (*a*) 0·022; (*b*) 0·056; (*c*) 0·149; (*d*) 0·301; (*e*) 0·757; (*f*) 2·000.
7.3. $\epsilon_{spec.} = 338{\cdot}8$.
7.4. (*a*) 0·0; (*b*) −1·15; (*c*) −5·35; (*d*) −8·08; (*e*) −15·15.
7.5. 700, 3,256.
7.6. 36·7.
7.7. DPNH $3{\cdot}33 \times 10^{-5}$ M; DPN^+ $1{\cdot}67 \times 10^{-5}$ M.
7.8. $1{\cdot}043 \times 10^{-5}$, $1{\cdot}043 \times 10^{-5}$, $1{\cdot}033 \times 10^{-5}$, $1{\cdot}055 \times 10^{-5}$.
7.9. *ortho* 0·14 g./100 ml., 4·9%; *meta* 1·55 g./100 ml., 53·8%; *para* 1·19 g./100 ml., 41·3%.
7.10. 87, 48·5, 8·5.
7.11. (1) $6{\cdot}25 \times 10^3$; (2) $6{\cdot}27 \times 10^3$; (3) $6{\cdot}11 \times 10^3$; (4) $5{\cdot}93 \times 10^3$ cm.^{2}/mole. The authors attribute the low value for TPN-isocitrate to the presence of *ca.* 5% impurity in the preparation.

CHAPTER VIII

8.1. (*a*) 1·61; (*b*) 1·59; (*c*) 1·43; (*d*) 1·70; (*e*) 1·55.
8.2. (*a*) 1·99; (*b*) 1·80; (*c*) 2·11; (*d*) 1·88.
8.3. (*a*) 1·55; (*b*) 1·82; (*c*) 1·46; (*d*) 1·37.
8.4. (*a*) 0·86; (*b*) 1·20; (*c*) 1·60; (*d*) 1·25; (*e*) 1·33; (*f*) 1·11; (*g*) 0·86; (*h*) 0·71; (*j*) 0·69.
8.5. O_2 159·6 mm.; N_2 593·2 mm. Hg.
8.6. $5{\cdot}78 \times 10^{-4}$; $1{\cdot}83 \times 10^{-3}$; $5{\cdot}78 \times 10^{-3}$; $1{\cdot}83 \times 10^{-2}$ M.
8.7. 76·9 µl./5 min.
8.8. $7{\cdot}5 \times 10^{-3}$ M; 0·099% (w/v).
8.9. $3{\cdot}33 \times 10^{-3}$ M.

8.10. Theoretical O_2 uptake for complete oxidation: 541, 1,082, 2,164, 4,328 μl. Per cent. theoretical O_2 uptake, endogenous not subtracted: 31·4, 30·4, 30·4, 29·9; endogenous subtracted: 21·6, 23·5, 25·6, 26·2. Endogenous suppressed completely in all except lowest concentrations.
8.11. 33·8% assimilated; endogenous respiration not suppressed by substrate.
8.12. Inhibits dissimilation of pyruvic acid. If KOH added to centre well, in presence of arsenite there should be no gas evolution.
8.13. 1·03, 1·57, 1·27, 5·00.
8.14. O_2 uptake: 200 μl.; CO_2 evolution: 201 μl. R.Q. 1·0.
8.15. Endogenous Q_{O_2}: 14·4, 7·45, 3·40, 1·65, 0·60. Glucose Q_{O_2}: 3·55, 4·75, 6·05, 7·70, 8·50.

CHAPTER IX

9.1. 18, 38, 80 min.
9.2. M.g.t. 35 min.; lag 50 min.
9.3. Unaerated 40 min.; aerated 32 min. Aeration began at 200 min.
9.4. M.g.t. 45 min.; 569 min.
9.5. Lag periods: control 400; malic 450; α-ketoglutaric 380; succinic 340; aspartic 260; glutamic 240 min.
9.6. M.g.t. of phenol and control cultures both 45 min., therefore no effect.
9.7. (*a*) 25; (*b*) 35; (*c*) 50 min.
9.8. 10-11 min.; 0·5 g. galactose/litre.
9.9. 450 mg. glucose and 550 mg. mannose/litre.
9.10. 104 min.

CHAPTER X

10.1. (*a*) 5·1%; (*b*) 73·1%.
10.2. 1:2.
10.3. −0·141 v.
10.4. +0·136 v.
10.5. −0·398 v.
10.6. 99·9% oxidized.
10.7. $n = 1$ for pH range 0-4; $n = 2$ for pH range 4-8; $E^\circ = 0{\cdot}386$ v.
10.8. −14·88, 26·04, 12·18; +20,300 cal., −17,760 cal., −8,307 cal.
10.9. 94·5%; r′H = 17·3.
10.10. −1,691 cal.; 79·93%.
10.11. −26,900 cal., −23,530 cal.
10.12. +0·136 v.; $n = 2$.
10.13. +0·796 v.
10.14. $pK = 6{\cdot}65$; $K = 2{\cdot}2 \times 10^{-7}$.
10.15. $E = 0{\cdot}82 + 0{\cdot}0148 \log p_{O_2}$; $p_{O_2} = 10^{-24}$ atmosphere.

CHAPTER XI

11.1. 0·0485 days^{-1}; 61·54; 37·93, 23·36, 8·68%.
11.2. 1,000.
11.3. 87·1 days; 0·00795 days^{-1}.
11.4. Haemoglobin, when incorporated into a red blood cell, does not take part in dynamic equilibria characteristic of other body proteins; it offers, therefore, a method for determining the life span of the red blood cell.
11.5. 1·262 g., 29%. N.B. The original article contains a typographical error and gives the excess density of water formed from leucine in the hydrolysate as 225 instead of 161 parts per million (personal communication, Dr. H. H. Ussing).
11.7. 3·418 and 3·026 g.

11.8. Tyrosine 3·0%, 11; phenylalanine 6·8%, 27; arginine 3·7%, 14; lysine 8·5%, 39; glutamic acid 8·6%, 39; aspartic acid 10·4%, 52; glycine 5·5%, 50; leucine 15·1%, 77.

11.10. Glutamic acid 19·1%, 55; aspartic acid 11·3%, 36; lysine 11·4%, 33; leucine 15·6%, 50; glycine 1·5%, 8.

11.11. Glycerophosphate. Average value : 6·75 hr.

11.12. β-C 940; α-C 10; carboxyl-C 10 counts/min./μmole phenylalanine. Glucose has 2,040 counts/min./μmole and the β-C has *ca.* half this activity. 3-Carbon fragment could therefore have come from triose-phosphate.

11.13. Glutamic acid 16·9%, 80; aspartic acid 9·71%, 51; tyrosine 4·78%, 18.

LOGARITHMIC TABLES

	0	1	2	3	4	5	6	7	8	9	1 2 3	4 5 6	7 8 9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	5 9 13 4 8 12	17 21 26 16 20 24	30 34 38 28 32 36
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4 8 12 4 7 11	16 20 23 15 18 22	27 31 35 26 29 33
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3 7 11 3 7 10	14 18 21 14 17 20	25 28 32 24 27 31
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3 6 10 3 7 10	13 16 19 13 16 19	23 26 29 22 25 29
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3 6 9 3 6 9	12 15 19 12 14 17	22 25 28 20 23 26
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3 6 9 3 6 8	11 14 17 11 14 17	20 23 26 19 22 25
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3 6 8 3 5 8	11 14 16 10 13 16	19 22 24 18 21 23
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	3 5 8 3 5 8	10 13 15 10 12 15	18 20 23 17 20 22
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2 5 7 2 4 7	9 12 14 9 11 14	17 19 21 16 18 21
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LOGARITHMS

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58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1 1 2	3 4 4	5 6 7
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66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1 1 2	3 3 4	5 5 6
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98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0 1 1	2 2 3	3 4 4
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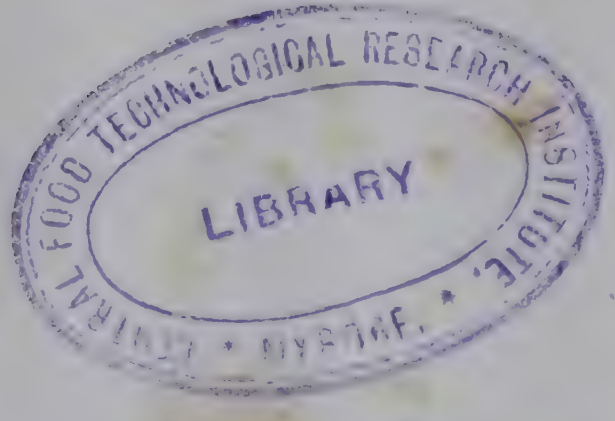

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